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The Effect of the Ionic Milieu on the Emergence of Radiocalcium from Tendon and from Sartorius Muscle

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In an effort to clarify the possible role of calcium in the functioning of skeletal muscle, our previous studies of Ca^{45} entry and exit with activity (Bianchi and Shanes, '59; Shanes and Bianchi, '60) and of Ca^{45} distribution (Shanes and Bianchi, '59) are now supplemented by an examination of the alteration of Ca^{45} escape from frog (*Rana pipiens*) tendon and muscles into non-radioactive media when the cations and anions of the Ringer's solution are modified. A preliminary report of some of these findings has been given (Bianchi and Shanes, '58).

METHODS

The release of Ca^{45} from individual tissues, previously exposed for several hours to normal modified Ringer's solution containing Ca^{45}, was followed as previously described (Shanes and Bianchi, '59), viz., by repeated exposure, usually for 10-minute intervals, to 2.5 ml of stirred and oxygenated non-radioactive solutions. One half ml of the 2.5 ml samples and of the acid extracts of the ashed residues, obtained after drying the tissues at the end of each experiment, was evaporated on planchets and counted; correction was made for self-absorption by having the same amount of salt present on all planchets. From these data the "desaturation" and "rate coefficient" curves were reconstructed (cf. Shanes and Bianchi, '59). The former describe the decline of the tissue radioactivity with time, measured as per cent of the initial, maximum activity. The latter give the time course of the average per cent *change* in activity per minute; this is estimated for each collection interval by dividing the activity appearing in the medium by the mean activity of the tissue during the collection and by the duration of the collection.

When the collection intervals are sufficiently short, the rate coefficient as defined is identical with rate constant in the case of the exponential release of radioisotope from tissues—as occurs when the cellular membranes are the chief barriers to diffusion and when the cells are identical in respect of geometry. But since the wide range of fiber sizes precludes the coefficient from being constant with time, the term "coefficient" is used instead of "constant." No connotation other than that of a *rate* of Ca^{45} escape relative to (i.e., divided by) the level of Ca^{45} in the fibers at the time should be construed for the phrase "rate coefficient." This measurement is employed because it is a sensitive indicator of alterations in the rate of escape of Ca^{45} and is independent of the progressive decline of the level of Ca^{45} in the fibers.

Changes in the ionic content of the milieu were made well after the rapid component of Ca^{45} escape was no longer evident, i.e., no earlier than two hours after wash-out.

All solutions contained cocaine at concentrations (usually 14 mg %) that eliminate spontaneous contractions such as tend to occur especially in media containing nitrate or lacking calcium (Bianchi and Shanes, '59).

The experiments were carried out chiefly in the Fall of 1958 at a room temperature of 25°C and on tissues removed the same day from the animals.

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RESULTS

Displacement of calcium by sodium. Muscles and tendons were exposed to Ca^{45} in a medium with 90% of the sodium replaced with choline. If sodium competes with calcium for the binding sites it should become apparent, after removal of the interfibrillar Ca^{45} in a calcium-free solution similarly low in sodium, as a transitory release of the radioisotope upon restoration of sodium to the medium.

Figure 1 shows the averaged results of two such experiments expressed as rate coefficients. These curves reveal a transitory release of Ca^{45} which is more marked for tendon. Hence, sodium competes with calcium for binding sites in tendon as well as in muscle. Comparison of the Ca^{45} release with our previous findings on self-exchange (e.g., fig. 2 in Shanes and Bianchi, '59) indicates that 85 mM sodium is about as effective as 1 mM calcium in the exchange with calcium in tendon, bu less than 0.2 as effective in muscle. I will be shown below that 0.3 mM calcium displaces all self-exchangeable calcium a well as 1 mM, hence calcium is even more effective than sodium and to an extent tha still cannot be fully stated.

Ca^{45} release by Ca^{40}. It was shown pre viously that less Ca^{45} emerges from muscl and tendon in a non-radioactive Ringer' solution lacking calcium than in one con taining the usual concentration of calcium (1 mM); this is particularly evident upon restoration of non-radioactive calcium Ca^{40}, to the medium after a two-hour wash out of Ca^{45} in calcium-free Ringer's, fo the retained Ca^{45} is rapidly released—abou as rapidly as from the extracellular space The time course of the release reveals tha this component of Ca^{45} is bound to super ficial sites and under our conditions ex changes with itself rather than with th

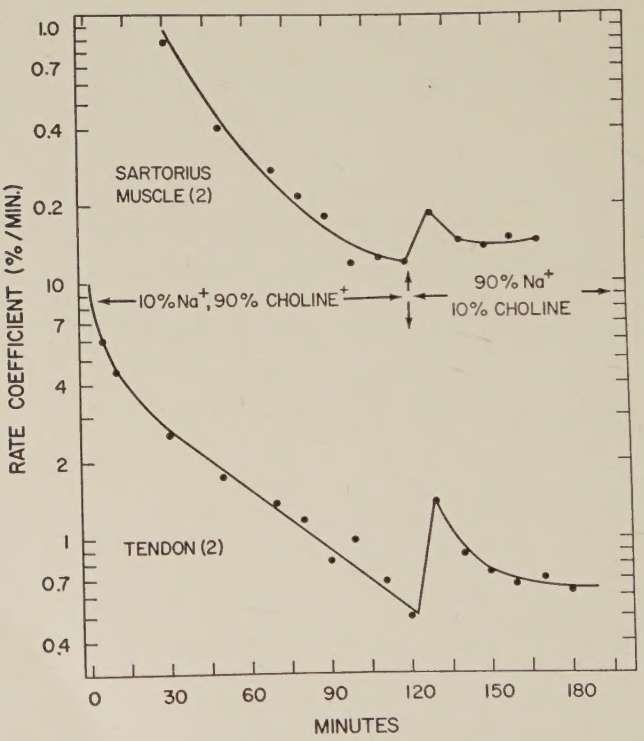

Fig. 1 The rapid transitory release of Ca^{45} from sartorius muscles and tendons of Achilles, measured as an increase in the rate coefficient, upon transfer of these tissues from a medium composed of 10% Na^+, 90% choline chloride to one containing 90% Na^+, 10% choline chloride. Preliminary exposure to Ca^{45} was also in the low sodium medium, but at a normal level of calcium.

ther ions of normal Ringer's solution. It s therefore referred to as "self-exchange-ible" calcium (Shanes and Bianchi, '59).

The uppermost curve in figure 2 is typical of the desaturation curves obtained earlier for muscle except that it shows the same percentage of self-exchangeable calcium (12% of the residual Ca^{45}) released when the calcium added to the calcium-free Ringer's brings the calcium concentration of the medium to 0.3 mM (i.e., 0.3 of the 1 mM usually present in our Ringer's solutions). That all the self-exchangeable calcium has indeed been displaced in 0.3 mM solutions is revealed by the absence in figure 2 of an additional release of Ca^{45} when the calcium concentration is subsequently raised from 0.3 to 1 mM. Such results are also obtained with tendon (fig. 3). We may conclude, then, that the sites that bind self-exchangeable calcium in muscle and tendon are saturated by calcium at a concentration 0.3 of that in normal Ringer's solution as well as by normal concentrations.

Figure 2 also demonstrates the effect of the time of exposure to Ca^{45} on the kinetics of washout obtained with muscles from the same batch of frogs. The number of preparations was limited but the results are internally quite consistent. It can be seen that the amplitudes of the slow components of the three desaturation curves in figure 2 vary with the equilibration time in Ca^{45} Ringer's, as might be expected from a greater uptake of radiocalcium by the fibers with time. Thus, the tangent to the three curves at the washout time of 120 minutes gives zero time intercepts of 19, 38 and 48% for the muscles soaked in Ca^{45} Ringer's for 15, 80 and 180 minutes respectively. The Ca^{45} spaces were, respectively, 0.27, 0.50 and 0.62 ml/gm. Since in our Ringer's solution the calcium concentration was 1 mM, the space figures represent the unidirectional transfer of cal-

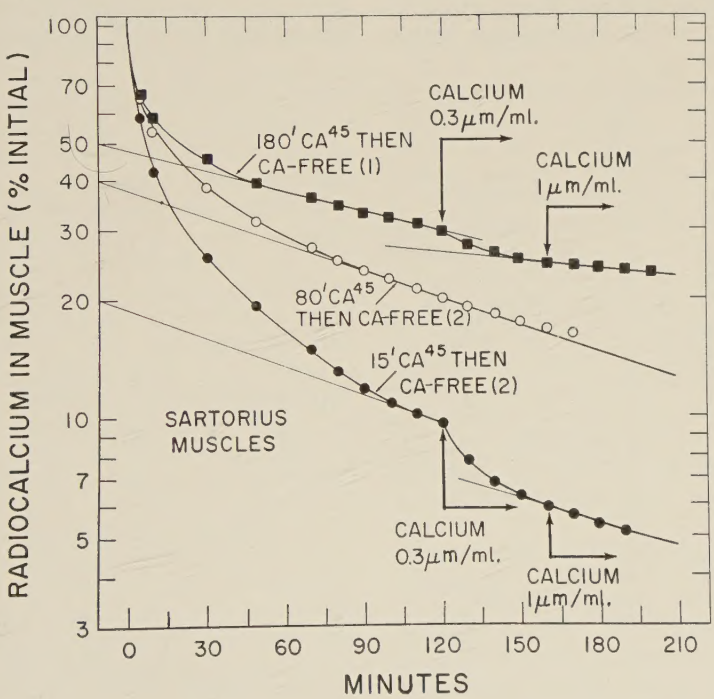

Fig. 2 The kinetics of decline of Ca^{45} in muscles exposed to radioisotope-free media after prior treatment for the indicated time to Ca^{45}-Ringer's solution. The effect of the addition of calcium to the washout media at two successive concentrations is shown for the lowermost and uppermost muscles. Numbers in parentheses give the number of preparations that provided each curve.

cium in μmol/gm. Consequently, the zero time intercepts represent, roughly, the amount of unidirectional transport of calcium into the fibers during equilibration, or 0.053, 0.19, 0.30 μmol/gm. These estimates can be regarded only as rough because of the numerous factors that prevent the desaturation curve from being strictly exponential (see Keynes, '54). The time constant obtained with these preparations, taken as that obtainable from the tangent lines at 120 minutes, is approximately 200 minutes—about half of that obtained with summer preparations (Shanes and Bianchi, '59). Using this time constant for the uptake of Ca^{45}, and taking 180 minutes equilibrated muscles as the reference, one would predict the fibers equilibrated for 15 minutes to contain 0.04 μmol/gm (compared to the extrapolation fig. of 0.053) and those equilibrated for 80 minutes to contain 0.16 μmol/gm (compared to the extrapolated fig. of 0.19). This is satisfactory agreement.

It appears then, that the fibers gain Ca^{45} during exposure to the radioisotope with a time constant approximating the time constant of Ca^{45} release in non-radioactive media. And since the final Ca^{45} "concentration" approaches that of the medium (Shanes and Bianchi, '59), it follows that the fluxes of calcium in both directions are approximately the same. The same conclusion can be drawn from an estimate of outflux from the calcium exchanged during long exposures to Ca^{45}, the time constant of washout (0.41 μmol/gm and 306 minutes in table 1), and a surface area of 300 cm^2/gm. This gives 0.074 μμmol/cm^2sec compared with earlier influx data of 0.072 and 0.094 μμmol/cm^2sec (Bianchi and Shanes, '59). Since intracellular calcium is probably bound (e.g., Harris, '57a; Niedergerke, '55), the mechanism whereby the efflux is maintained remains to be resolved.

Two additional features of the desaturation curves in figure 2 may be noted: (a) The shorter the prior exposure to radiocalcium the later is completed the initial fast component of Ca^{45} release and (b) the percentage of Ca^{45} released by Ca^{40}, ex

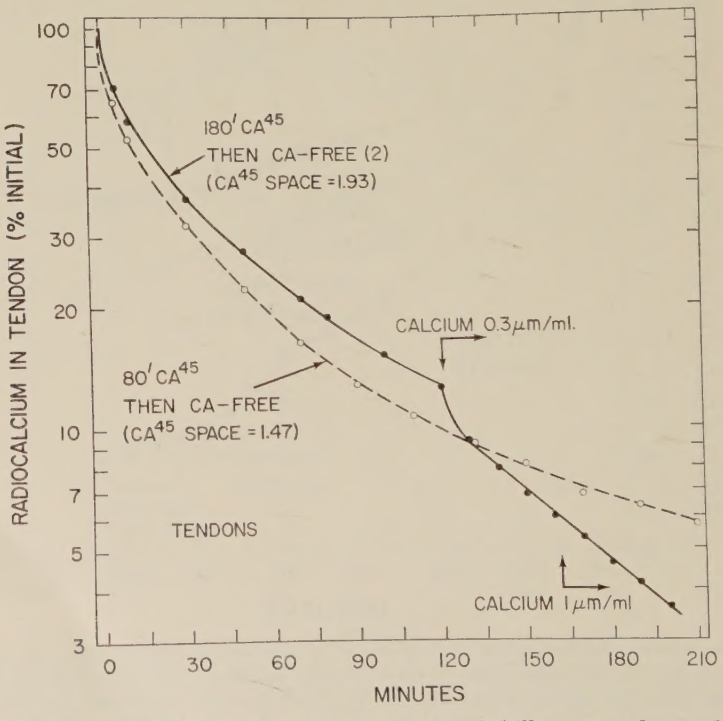

Fig. 3 The kinetics of decline of Ca^{45} in tendons of Achilles exposed to radiosotope-free media after prior treatment for the indicated times to Ca^{45}-Ringer's solution. As in figure 2.

pressed relative to the residual Ca^{45} at the time Ca^{40} is added, is greater for muscles equilibrated a shorter time. The second observation is not surprising inasmuch as the self-exchangeable calcium is derived from calcium bound to surface sites, which should be close to equilibrium with the medium for all equilibration times, whereas the slow component, from the fibers, will be appreciably smaller the shorter the exposure to Ca^{45}. However, while the percentage release of Ca^{45} in Ca^{40} solutions is larger—24% instead of 12%—it is still far from as large as expected. Thus, 12% in the uppermost preparation of figure 2, subjected for three hours to Ca^{45}, corresponds to 0.022 μmol/gm of self-exchangeable calcium, while 24% in the former represents only 0.0062 μmol/gm (the absolute values are obtained by multiplying together the Ca^{45} spaces, the per cent change in radioactivity at the time Ca^{40} is added, and the calcium concentration of the Ringer's, viz., 1 mM). The much smaller figure for short equilibration time could be a consequence of exchange of part of the surface Ca^{45} with Ca^{40} in the myoplasm, where the specific activity is very low, during the washout period in non-radioactive Ringer's solution. Such an effect would be much smaller or negligible when the specific activity of the myoplasm approaches that of the medium as a result of long exposure to Ca^{45} Ringer's solution.

The greater delay in the completion of the fast component of Ca^{45} release after brief exposure to "hot" Ringer's solution may also be related at least in part to the penetration of Ca^{45} into the fibers from more superficial binding sites. Thus, as Ca^{45} enters, it may be expected to be deposited as a cortical ring in the periphery of the myoplasm, a ring that gradually extends more deeply towards the axis of the fibers with continued equilibration in Ca^{45}. With brief exposures to the radioisotope, the Ca^{45} in the myoplasm will be chiefly near the periphery so that it declines during washout by diffusing into the protoplasm as well as into the Ca^{45}-free medium. Hence the gradient normal to the axis would continue to fall longer than when the deeper regions are more thoroughly filled by long soak periods. The very slow, nearly exponential phase may be presumed to appear when the gradient in the myoplasm is continuously downward from the axis to the periphery of the muscle fibers; such an exponential loss occurs at later times during diffusion from a homogeneous cylinder. Harris ('57b) provides a quantitative approach to the kinetics of this type of situation.

Replacement of Cl^- with NO_3^-. This change in the medium has been shown to augment the calcium entry per twitch in proportion to the increase of twitch height without significantly increasing the passive entry of calcium (Bianchi and Shanes, '59). Since indirect data suggest that the calcium which enters with activity is derived from binding sites saturated with calcium in chloride Ringer's solution (Bianchi and Shanes, '59; Shanes, '58) and a hypothetical basis has been offered for the nitrate effect in terms of a modification in the binding of calcium (pp. 251–257 in Shanes, '58), the experiments to be described were carried out to determine whether nitrate may indeed alter the binding of calcium.

This effect was sought in the two components of Ca^{45} escape that had been distinguished previously, viz., that which appears as part of the rapidly escaping fraction in calcium-free Ringer's solution and is designated as "removable," and that which emerges briefly and rapidly upon restoration of calcium to the medium after the "removable" calcium is absent i.e., the "self-exchangeable" calcium (Shanes and Bianchi, '59). A comparison was carried out on the escape of Ca^{45} from muscles in which one of a pair had been soaked in Ca^{45} chloride Ringer's solution and the other in equally radioactive nitrate Ringer's solution. The release of Ca^{45} was then followed in non-radioactive solutions containing the same anions as before, usually with calcium lacking so that the self-exchangeable calcium could be evaluated later by the addition of 1 mM calcium. Several tendons were examined similarly to determine whether nitrate effects were any more specific for muscle and hence attributable to the fibers rather than to the connective tissue.

Typical experimental curves obtained with tendon and muscle are shown in figure 4. Data calculated from such curves

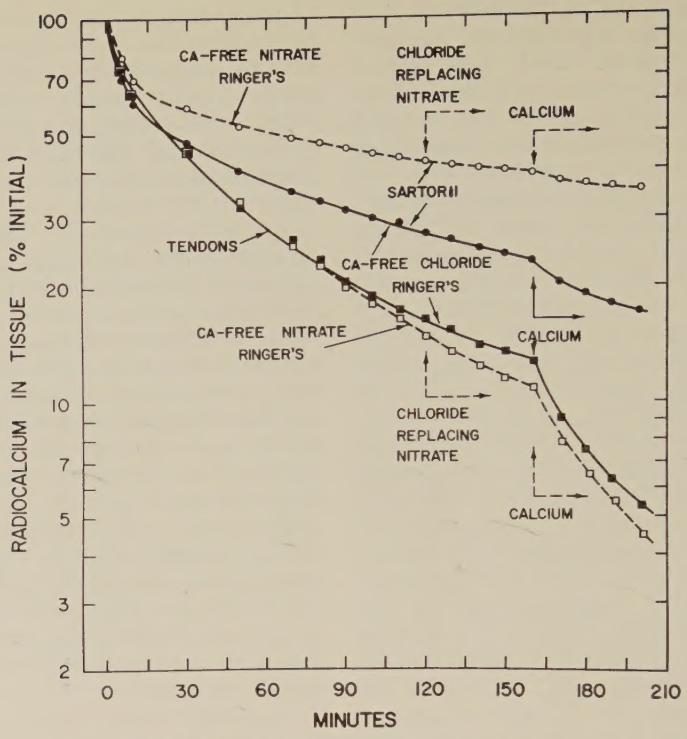

Fig. 4 A comparison of the desaturation curves of paired tendons and muscles after one of each pair had been equilibrated for 4 hours in "hot" chloride Ringer's solution and the other in "hot" nitrate Ringer's solution. Washout media contained the same anion as that employed during Ca^{45} treatment but lacked calcium until added as indicated. Chloride replaced nitrate in the washout solutions 40 minutes before the addition of calcium.

for muscle are summarized in table 1. The results show that the emergence of Ca^{45} from tendon is unaffected by the change in the anion of the medium, whereas certain differences are clearly apparent in muscle. Thus, in the three pairs of tendon examined, the Ca^{45} level of the tissues after 120 minutes of Ca^{45} washout was less in NO_3^- in two cases by 1.2 and 0.7%, and greater by only 3.2% in the third. On the other hand, extrapolation of the slow, nearly exponentially declining portion of the desaturation curves of muscle reveals that the zero time intercepts are smaller in Cl^-. As may be seen in table 1, the fast component is 45% in NO_3^- and 56% in Cl^-. The difference, which is significant, remains constant; for example, at 120 minutes the average remaining radioactivity is $15 \pm 1.4\%$ higher for muscles in NO_3^- than for their mates in Cl^-, which is not significantly different from the difference of $12 \pm 4.3\%$ obtained similarly at zero time.

The percentage data do not fully describe the situation. They are converted in table 1 to μmol/gm by the multiplication by "Ca^{45} space" (i.e., the ml of medium that contains the same radioactivity as 1 gm of muscle) and by the calcium content of the medium, viz., 1 μmol/ml.

The results of these calculations, tabulated in column E′ and E, indicate no significant difference in the initial amount of calcium exchanged with the cell interior during the exposure to Ca^{45} in NO_3^-, but 0.16 μmol/gm less calcium emerges quickly during washout. Also noteworthy are the substantially longer time constant of Ca^{45} emergence in NO_3^- and the somewhat smaller Ca^{45} space that is on the borderline of significance. If the latter is regarded as genuine, it represents a deficit of 0.0 μmol/gm compared to the over twofold

TABLE 1

Comparison of desaturation curve characteristics—the initial total Ca^{45} space, the parameters of the slow component (viz. the zero time intercept, F′, its equivalent calcium content, E′, and the time constant, τ) and the corresponding data for the fast component (F, E)—and of the self-exchangeable fraction, X, of paired muscles subjected to NO_3 and Cl Ringer's solutions

Ca^{45} space		F′		E′		τ		F[1]		E		X	
NO_3	Cl	NO_3	Cl	NO_3	Cl	NO_3	Cl	NO_3	Cl	NO_3	Cl	NO_3	Cl
ml/gm		%		*μmol/gm*		*min.*		%		*μmol/gm*		*μmol/gm*	
0.77	0.74	44	35	0.34	0.26	350	251	56	65	0.43	0.48	0.019	0.009
0.81	0.88	54	49	0.44	0.43	855	442	46	51	0.37	0.45	0.008	0.020
0.89	1.05	59	48	0.53	0.50	457	202	41	52	0.36	0.55	0.022	0.042
1.00	1.03	75	46	0.75	0.47	422	322	25	54	0.25	0.56	0.030	0.031
0.79	0.93	48	40	0.38	0.37	388	311	52	60	0.41	0.56	0.022	0.023
Av. 0.85	0.93	56	44	0.49	0.41	494	306	44	56	0.36	0.52	0.020	0.025
Av. diff.[2]	0.074		−12		−0.08		−188		12		0.16		0.0048
S.E.[3]	0.032		4.3		0.05		64		4.3		0.046		0.0051

[1] The fraction of the emerging Ca^{45} escaping as the fast component (=100−F′).
[2] Average difference based on Cl−NO_3 of individual pairs.
[3] Small sample S.E. of the average difference of the pairs.

larger deficit given by the fast components. These deficits are not attributable to a smaller extracellular space, for in a separate series of measurements of sucrose space no consistent difference was found. Thus, in 6 muscles in nitrate this was found to be 0.22 ± 0.02 ml/gm compared to 0.21 ± 0.03 in the control muscles.

In figure 4 it may be seen that replacement of the NO_3^- by Cl^- at late times in preparations previously subjected to NO_3^- does not alter the release of Ca^{45}. In the same figure it appears that NO_3^- has reduced the relative amount of self-exchangeable calcium. However, the data in column X of table 1 show that this was not a consistent effect and in fact that the self-exchangeable calcium is unaltered by nitrate.

DISCUSSION

Of particular interest are the results with nitrate, for these are the first we have found to demonstrate a difference between muscle and tendons sufficiently distinct to allow one to attribute the site of action unequivocally to the fibers.

The best interpretation of the findings is not the most direct. Thus, ordinarily the longer time constant in nitrate would be regarded as the consequence of reduced efflux (e.g., because of a decrease in permeability to calcium) and the smaller fast component of emergence as due to a smaller interfibrillar space. However, the latter has already been noted as inconsistent with an unchanged sucrose space. The former, if due to a change in permeability (a sustained change in potential is not produced by NO_3^- according to Hodgkin and Horowicz, '58), leaves unexplained the absence of the change in influx indicated by column E′. Thus, had a change occurred in permeability as large as that indicated by the time constants in table 1, the unidirectional uptake in nitrate would have been ⅓ less than in chloride; the nitrate figures actually tend to be larger than for chloride.

A more satisfactory interpretation is the following: The longer time constant in nitrate is only apparent. It is actually due to a certain amount of Ca^{45} being permanently retained by the fibers, the deficit of rapidly emerging Ca^{45} being a reflection at least in part of this fixed calcium.

This view is actually simpler than the first in that two phenomena associated with nitrate action are now linked together. Moreover, it can be shown that the quantitative aspects are also largely accounted for. A rough estimate of the apparent change in time constant due to permanent fixation of some of the superficial calcium is obtained by assuming for convenience that intrafibrillar Ca^{45}, Ca_t, emerges in chloride and nitrate Ringer's solution according to the simple exponential

$$Ca_t/Ca_0 = e^{-t/\tau} \qquad (1)$$

Ca_0 is the Ca^{45} inside the fibers initially, i.e., at $t = 0$, τ is the time constant. The loss of Ca^{45} from muscles, exposed to radioactive solution for several hours, as in the nitrate experiments, only approximates an exponential process (Shanes and Bianchi, '59; also see fig. 4). Let A be the amount fixed to the fibers by nitrate. If we now attempt to evaluate a new time constant, τ', in the usual fashion, that is, by measuring the time at which the Ca^{45} of the muscle fibers is 1/e of that present initially, we are obtaining the time, τ', when the calcium in *and on* the fibers is

$$(1/e)\,(A+Ca_0) = A+Ca_{\tau'} = A+Ca_0\,e^{-\tau'/\tau} \qquad (2)$$

Hence

$$\tau'/\tau = \ln\,(1/(0.37 - 0.63A/Ca_0)) \qquad (3)$$

In anticipation of the later demonstration that A is overestimated by the data in column E, table 1, we assign A the value 0.1; Ca_0 is taken as 0.41 from the same table. We thus obtain $\tau'/\tau = 1.53$ compared to the experimentally obtained ratio of 1.62. A slightly larger value of A, e.g., 0.11, will give the actual experimental ratio; the deviation is well within the range of variability of the data.

If this approach is correct, it should account also for the intercepts indicating an initial Ca^{45} content in NO_3^- that does not give as large a value for A as it should. If we examine E′ carefully, we note that all the NO_3^- figures are actually larger, although by no means to the extent to be expected from the above estimates for A.

It will now be shown that the fixed component would be less apparent by virtue of the extrapolation procedure used to obtain the zero time intercepts. Thus, let us take as correct the zero time intercept of the desaturation curve in chloride Ringer's solution—equal to 0.41 μmol/gm. The level of saturation, S, at 120 minutes, the region from which the extrapolation lines were drawn, is given by the following equation because τ is 306 minutes:

$$S = 0.41\ e^{-120/306} = 0.28 \qquad (4$$

To this may be added the true deficit, say 0.10, in the fast washout component found in NO_3^-. Hence, at 120 minutes the saturation level in NO_3^- is 0.38 μmol/gm. The apparent intercept, y, obtained with the extrapolation procedure we have used is therefore given by

$$0.38 = ye^{-120/494} \qquad (5$$

since 494 is the apparent time constant in NO_3^-. Therefore, $y = 0.48$ μmol/gm which is larger by only 0.07 than the intercept in chloride instead of by the expected 0.10. Thus, the zero time intercept is too small by .03, which would have contributed to making the larger intercept in nitrate less readily demonstrable. By the same token, the deficit estimated from the rapid components of Ca^{45} washout will be too large by about 0.03 μmol/gm, giving a total apparent deficit of 0.13 compared to our experimental figure of 0.16.

Within the variability of our data, then the quantitative and qualitative aspects of our findings appear satisfactorily accounted for in terms of the fixation by nitrate of about 0.1 μmol/gm calcium at the superficial sites on muscle fibers where ionic exchanges normally occur readily. Self-exchangeability, on the other hand, is not affected by the nitrate. If our interpretation is correct, the absence of a quick release of calcium upon the return from nitrate to chloride Ringer's solution (fig. 4) indicates that the fixation by nitrate is not readily reversed.

The figure of 0.1 μmol/gm is identical with our estimate, based on corrections for binding by connective tissue, as to the amount of non-selfexchangeable calcium bound to muscle sites (table 3 in Shanes and Bianchi, '59). It is rather remarkable that all such calcium should have been fixed by the nitrate. This appears to provide an independent validation of our use of tendon as a model of the calcium binding characteristics of the connective tissue in frog sartorius muscle.

The suggestion was put forward earlier, ased on the known potency of the bind- ng of anions by serum albumin, that the nhancement of twitches when other halo- ens or nitrate replace chloride may be due n part to improved binding of calcium to nuscle which in turn contributes to en- anced calcium entry (p. 254 in Shanes, 58). The enhanced entry has already een found (Bianchi and Shanes, '59). A nechanism whereby binding may be trengthened is as follows: cationic groups n the vicinity of anionic sites in the nuscle membrane can be expected to epel the calcium attracted to the anionic roups, thereby weakening the force of alcium binding. Anions in the medium vill be attracted to the cationic groups and hereby will tend to reduce the repulsion f calcium; those attracted more strongly nd more closely to the cationic groups nay be expected to be more effective in trengthening the binding of calcium. his provides a basis for the parallelism etween the binding of anions to serum lbumin (e.g., Scatchard et al., '57) and heir ability to augment the twitch (Kahn nd Sandow, '55).

It is unlikely that all of the 0.1 μmol/gm alcium which has been estimated to be on he muscle membranes is concerned with he contractile process. If, as a first ap- roximation, the calcium transfer induced luring potassium contracture is considered s an indication of the total calcium ctually concerned with contraction (Bi- nchi and Shanes, '59), we can conclude hat only about 0.2 of the superficial fiber alcium is related to the mechanical activ- ty of muscle. If this calcium were spread niformly over the fiber surfaces, the dens- ty would be relatively low (ca. 1 calcium on every 17 A). But attention has been alled to the more likely situation that in mphibian muscle fibers calcium con- erned with contraction may be released rom "tubules" in the vicinity of the z line p.256 in Shanes, '58; Bianchi and Shanes, 59; Shanes and Bianchi, '60); an esti- nate of the calcium density in such tubules equires histological data which are as yet navailable.

The slightly smaller total Ca^{45} space of itrate-treated muscles is not accounted or by the above considerations. There are at least two possibilities. The influx of calcium may actually be smaller in nitrate, possibly for the same reason that KNO_3 enters muscle more slowly than KCl (Conway and Moore, '45) or because the fixation of the calcium by nitrate interferes with transport through the membrane. Some of the calcium normally bound to the fibers may be unable to bind in the presence of nitrate, but this seems unlikely in view of the opposite effect indicated by our data. A third possibility—a smaller interstitial space in nitrate—need not be considered in view of our finding that the sucrose space is unchanged by nitrate treatment. If the first interpretation is indeed the correct one, it offers an additional basis for the questionable increase in the absolute amount of calcium exchange indicated by the zero time intercept of the nitrate preparations, i.e., a somewhat smaller protoplasmic exchange tends to mask that fixed to the surface since the sum of these contributes to the intercept. Measurements on single muscle fibers might provide the sensitivity needed to establish the reality of an effect by nitrate on calcium influx.

It is of interest that the sites occupied by self-exchangeable calcium are saturated at normal and at ⅓ normal calcium concentrations in the medium. It will be recalled that the entry of calcium during stimulation, unlike penetration in resting muscle, also shows saturation in that it is not increased by trebling the calcium content of the medium (Bianchi and Shanes, '59). It would be of interest to determine whether the calcium level of the medium at which the degree of saturation of self-exchangeable calcium is affected is the same for the calcium that enters during activity. In any case, the high affinity for calcium is noteworthy, for it may be an important factor with respect to which muscles differ and thereby differ in their sensitivity to the depletion of calcium in the medium. This difference may be expected to be particularly evident in muscle fibers of the heart where, in contrast to the situation with skeletal muscle, contractility is obviously related to the calcium level of the medium (Reiter, '58). This is cur-

rently under study in our laboratory by Dr. S. Winegrad.[2]

SUMMARY

Sodium can displace calcium from the superficial sites in both tendon and muscle. The time course of the gain of Ca^{45} by muscle fibers in Ringer's solution containing this isotope mirrors the subsequent loss to non-radioactive media. The superficial calcium-binding sites at which Ca^{45} exchanges with Ca^{40} are saturated in both muscle and tendon at calcium concentrations of 0.3 as well as of 1 mM. Muscle differs from tendon in that nitrate prevents part of the Ca^{45} that rapidly emerges in non-radioactive solutions from appearing. The calcium retained by nitrate appears to be that previously estimated to be located on the surface of the fibers.

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[2] Since submission of this manuscript Win grad ('60) has established a linear relation b tween contractility and calcium entry per bea

Leakage of Phosphate Compounds from Ultraviolet-Irradiated Yeast Cells

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One of the interesting but not frequently studied aspects of photobiology is the phenomenon of the loss of cellular materials to the medium following irradiation of cells with ultraviolet light. In 1937, Fardon, Norris, Loofbourow and Ruddy reported that when the cell-free supernatant from ultraviolet-irradiated yeast cells is added to a suspension of normal cells, there results a 50% increase in the respiratory activity of these cells. Loofbourow, Cook and Simpson ('38) found that the supernatant of the irradiated cells also contains factors which promote normal yeast cell proliferation. Studies by Adelstein, Hershey, Loofbourow and Sizer ('52) demonstrated that the stimulated oxygen consumption follows the same time course as of the corresponding growth increase. The stimulating fluid was analyzed chemically and spectrophotometrically by Loofbourow et al. ('38) and it was found to contain phosphorus, nitrogen and pentose, but no halogens, sulfur, deoxypentose or protein. They also reported that the absorption spectrum of the supernatant shows a peak at 260 mμ indicating that the supernatant contains nucleic acid derivatives.

The availability of radioisotopes coupled with an interest in the actions of radiations on living cells led Hevesy and Zerahn ('46) to do experiments in which they tested the effects of x-rays and ultraviolet radiations on the permeability of yeast to P^{32}. They grew cells on medium containing P^{32} and irradiated the washed cells. Little P^{32} left the cells which were irradiated with x-rays when measured after 24 and 48 hours. Ultraviolet radiations caused a large amount of phosphate, including acid-soluble phosphate, to leak into the medium. As in the case of x-rays, the measurements were made 24 and 48 hours after irradiation was accomplished.

This paper deals with time-course studies of the leakage of P^{32} from uniformly-labeled ultraviolet-irradiated yeast cells, the influence of physical and chemical variables on this process and with a chromatographic study of the leakage products.

MATERIALS AND METHODS

A strain of *Saccharomyces cerevisiae* originally isolated at the University of Toronto was used for these experiments. The yeast was grown in 125-ml Erlenmeyer flasks in 25 ml of liquid medium; the medium contained 1.0% yeast extract and 2.0% dextrose. For the radioactive experiments, cells were uniformly labeled with P^{32} by growing them in medium to which was added 25 microcuries of P^{32} as H_3PO_4. Inoculation was made with a loop from a slant culture 24 hours old. The inoculated culture flasks were shaken on a wrist-type shaker for 24 hours at 30°C. Following growth, the cells were washed 8 times with distilled water and suspended in water or buffer solution to give a concentration of 1.5×10^8 cells per ml. For some of the experiments the cells were suspended in water because paper chromatography work on the leakage materials was to be carried out using similarly-treated samples. The presence of salts such as those in phosphate-buffer solutions seriously interferes with chromatographic separations. For the time-course irradiation studies with P^{32} labeled cells, 5 ml of a suspension of cells in water were irradiated in a shallow dish with an inside diameter of 45 mm and depth of 14 mm using

[1] Research carried out at Brookhaven National Laboratory under auspices of the United States Atomic Energy Commission.

a Hanovia utility model ultraviolet lamp. This lamp has a high pressure mercury arc with a maximum output at 336 mμ with 17% of the output in the region 220 to 280 mμ. The characteristics of the lamp have been described in more detail elsewhere (Swenson, '58). The lamp was operated without a filter 57 cm above the surface of the dish. The exposed surface area of the suspension was small enough that all of the surface was uniformly irradiated. During irradiation the yeast suspension was kept in motion with a magnetic stirrer. The intensity of the lamp at the surface of the suspension was 195 ergs/mm²/sec. as measured with a Hanovia ultraviolet meter.

Following irradiation the cell suspension was transferred quantitatively to a 125-ml Erlenmeyer flask and diluted to 25 ml. The flask was capped with aluminum foil and mounted on an oscillating shaking mechanism with the flask immersed in a 30°C water bath. The flasks were protected from visible light by a black curtain to prevent photoreactivation from occurring. At intervals, 1.5 ml of the suspension were withdrawn and the cells were packed by centrifugation. One ml of the cell-free supernatant, containing the products which leaked from the cells, was withdrawn and deposited on an aluminum planchet 1¼ inch in diameter. The planchets were dried under an infrared lamp and counted using a G.M. counter and scaler. The dry weight of 1 ml of the undiluted yeast suspension was 3.3 mg. Leakage from the diluted suspension is expressed as counts per minute from 0.66 mg of cells. The number of counts of P^{32} in the washed diluted cells prior to irradiation was determined by counting a planchet on which 1.0 ml of the suspension had been deposited and dried.

For the experiments testing the influence of the pH on leakage, buffer solutions having various pH values were prepared from 0.033 M solutions of H_3PO_4, KH_2PO_4 and K_2HPO_4; at the basic end of the pH scale 1 N KOH was added to the KH_2PO_4 solution to adjust the pH to the desired value. The cells were incubated for 20 minutes in the appropriate buffer before irradiation; following irradiation, the cell suspension was diluted to 25 ml with the buffer and incubated in the usual manner.

For the experiments using glucose the cell were irradiated in phosphate buffer, pH 6.7. The irradiated suspension was then diluted with buffer containing glucose to give a final concentration of 0.03 M.

The poison experiments with 2,4-dinitrophenol and sodium azide were carried out in phosphate buffer, pH 4.5. The cells were irradiated for 7½ minutes at pH 3.5 and then diluted to 25 ml with a solution containing the poison at the same pH; the final concentration of the sodium azide and dinitrophenol was 0.001 M.

The concentrates of leakage products for paper chromatographic analysis were prepared by irradiating for 20 minutes, 25 ml suspensions of unlabeled or uniformly-labeled cells suspended in water, followed by gentle shaking for a two-hour period at 30°C. The cells were then centrifuged and the supernatant was decanted. The supernatant was passed through a Seitz filter to insure that the solution was cell free. The leakage products were then concentrated by lyophilization to give a final volume of 0.3 ml. The resulting solution was an amber color with a flaky, white precipitate which was removed by centrifugation.

Ten to 25 microliters of the concentrate were used for paper chromatographic analysis. The concentrate was applied to the corner of filter paper with a micropipette and dried with a jet of filtered compressed air. The spots after drying were about ½ inch in diameter and gave approximately 100,000 counts per minute when measured with a gas-flow counter described by Fuller ('56). Whatman no. 4 paper, 18¼ by 22½ inches, was used. Before using for chromatography, the paper was washed batchwise with 1% oxalic acid in a large plexiglass filter and rinsed with distilled water until the pH was neutral (Hanes and Isherwood, '49). The packet of 100 papers was then dried at 50°C in a Reco chromatographic drying oven. Descending chromatography was employed following the method of Benson et al. ('49) in which water-saturated phenol was the solvent for the long dimension and butanol-propionic acid was used for the short dimension.

Autoradiographs of the chromatograms were made by placing the chromatograms in contact with 14 by 17 inch Eastman No-Screen x-ray film for several days (Ber

on et al., '49). For identification work, adioactive spots were eluted with water nd co-chromatogrammed with authentic ompounds using tertiary butanol-water-icric acid as a solvent system (Hanes nd Isherwood, '49). The resulting chro-natogram was sprayed with Hanes-Isher-vood acid molybdate reagent (Hanes and sherwood, '49) to develop a blue spot haracteristic of a phosphorus-containing ompound. If the blue spot on the chro-natogram coincided exactly with the black pot on the radioautograph, identity was ssumed to be established. For the nu-leotide work, two-dimensional chromatog-aphy was carried out using unwashed Vhatman no. 1 paper and employing am-nonium sulfate and isopropanol (Paladini nd Leloir, '52) for the long dimension of he paper and ammonium acetate-ethanol Hall and Khorana, '54) for the short imension. The use of these solvents al-owed nucleotides to be located by ultra-iolet absorption. Inorganic P^{32} orthophos-hate produced a large black area on the utoradiographs of such chromatograms, hus obscuring the field normally occupied y the nucleotides. This complication was ealt with by first adjusting the 25 ml of eakage solution to pH 5.5 with 1.0 N HCl nd then slowly drawing the solution hrough a bed of charcoal, Norite A, in a mall Buchner funnel (Hurlburt, '57). At his pH nucleotides are adsorbed but or-hophosphate and sugar phosphates are ot. The charcoal was then washed 4 imes with 10 ml volumes of distilled vater. The adsorbed nucleotide materials vere eluted from the charcoal by slowly rawing through the bed 40 ml of 50% thanol followed by 40 ml of 50% ethanol lus 1% concentrated NH_4OH. The eluent vas concentrated as before by lyophiliza-ion. A small amount of this concentrate vas chromatogrammed along with au-hentic nucleotides. The concentrate was potted to provide sufficient radioactivity o give good radioautographs (20,000 /m), but with insufficient total material o give absorption spots when viewed with n ultraviolet source. The authentic nu-leotides were spotted in such quantities 10 to 20 μ liters of 0.025 M solution) to roduce a definite dark spot when viewed vith an ultraviolet source. After chro-matography and autoradiography, the areas of ultraviolet absorption were out-lined on the chromatogram. If the absorption area of the authentic compound coincided with a dark spot on the film, the authentic compound was concluded to be present in the leakage material.

Amino acids on the chromatograms were detected by spraying with a solution of 0.5% ninhydrin in ethyl alcohol with a trace of collidine. The colors were devel-oped by heating the chromatograms in a drying oven for one minute at 100°C.

The free phosphate components of non-irradiated cells were isolated by successive extraction with boiling-hot 80%, 60% and 20% ethyl alcohol. The extracts were com-bined and concentrated under vacuum for chromatography.

The temperature coefficient experiments were carried out in temperature-regulated water baths except for the one at 5°C. In this case, the sample was shaken on a platform shaker in a constant-temperature room regulated at 5°C.

The high temperature experiments were carried out as follows: A 12 ml tapered centrifuge tube containing 5 ml of washed, labeled cells was im-mersed for a desired period of time in a water bath maintained at 55°C. The sus-pension was stirred when the tube was first immersed to insure rapid attainment of maximum temperature. Cell viability after irradiation was determined by taking aliquots of diluted cell suspensions and mixing them with warm agar growth me-dium in a petri dish. The medium con-tained 2.0% glucose, 1.0% yeast extract, 2.2% agar in 0.05 M KH_2PO_4. The latter was added to lower the pH and minimize bacterial growth. Colony counts were made after the plates were incubated for three days at 30°C.

Spectrophotometric work was carried out with a Beckman DU quartz spectro-photometer.

RESULTS

It is seen in figure 1 that a negligible amount of radioactive material leaks from yeast cells suspended in water over a 24-hour period. Low dosages of ultraviolet irradiation for 5 and 7½ minutes cause slow, continuous leakage of phosphate-

containing compounds during the same period. Increasing the dosage increases the rate and total amount of leakage. The maximum rate of leakage occurs with 20 minutes exposure. For this irradiation time and for 30 minutes, the leakage is very rapid for approximately 4 hours and then ceases. Labeled phosphate compounds having 4000 counts per minute leak from one ml of cells in the 4-hour period. One ml of cell suspension dried on a planchet gave approximately 8000 counts per minute; this means that about 50% of the labeled phosphate in the cell is lost in this period. When the rate of initial leakage is calculated from the data in figure 1 and plotted against the exposure time, the curve seen in figure 2 is obtained. This curve shows that the rate of leakage is relatively less at low dosages. It should be noted at this point that the terms low and high dosages are only with respect to the leakage phenomenon. All of the dosages used in these experiments are lethal when ability to form colonies is considered. With 5 minutes exposure 0.1% of cells survive. Several other facts are appropriate to mention at this point. Microscopic examination revealed no visible damage to the cells and hemocytometer counts showed the same number present in suspensions before and after irradiation.

The effect of irradiating cells in water followed by suspension in phosphate buffers having different pH values was tested. For these experiments, the cells were in

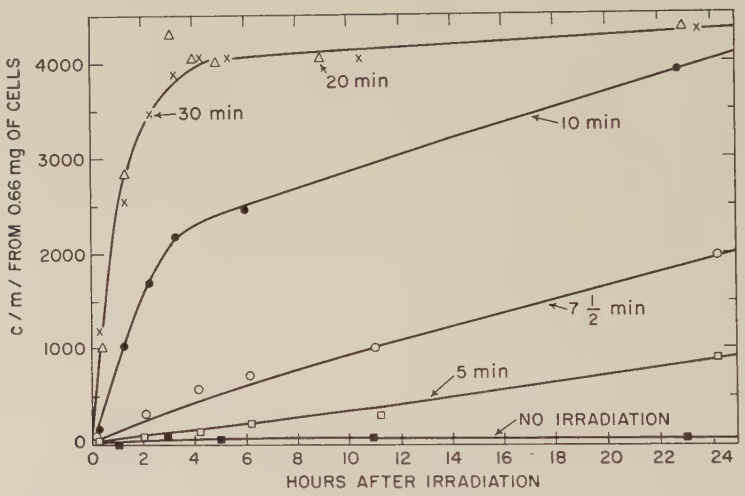

Fig. 1 Time-course curves for leakage of P^{32} from cells irradiated for various lengths of time.

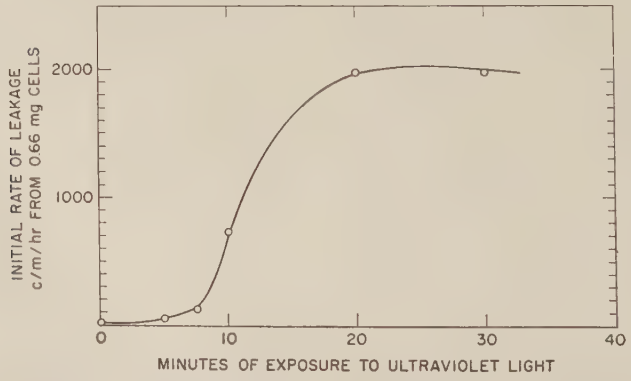

Fig. 2 Effect of time of exposure to ultraviolet radiations on the initial rate of leakage.

adiated for 5 minutes, a dosage causing nly a limited amount of leakage from ells suspended in water. The results, seen n figure 3, demonstrate that the pH of he medium strongly influences the rate nd amount of leakage of phosphate com- ounds from the cells. It may be noted pon comparing figures 1 and 3 that the eakage from cells at pH 6.7 is approxi- nately the same as for those irradiated for 5 minutes suspended in water. Figure 4, n which the initial rate of leakage is lotted against pH, shows that the ptimum pH for the cells, i.e., the pH at hich the cells lost the least amount of phosphate, is in the region of 6.0. The controls for this experiment are seen in the lower half of figure 5. These curves demonstrate that within the pH range of 2.6 to 8.7 a slight amount of leakage does occur from non-irradiated phosphate-buffered cells and that the pH of the medium influences radiation-produced leakage. At pH values above 8.7 and below 2.6, according to the data plotted in the upper curves of figure 5, phosphate leaks from non-irradiated cells in considerable quantities.

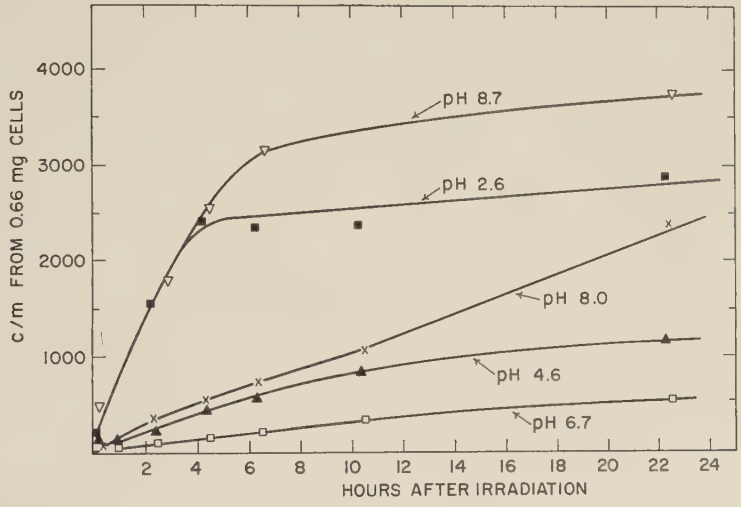

Fig. 3 Time-course curves for leakage from irradiated cells in phosphate buffers with different pH values. Irradiation time, 5 minutes.

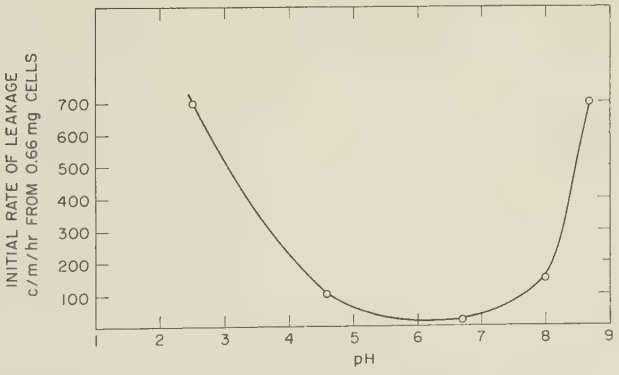

Fig. 4 Effect of pH on initial leakage rate.

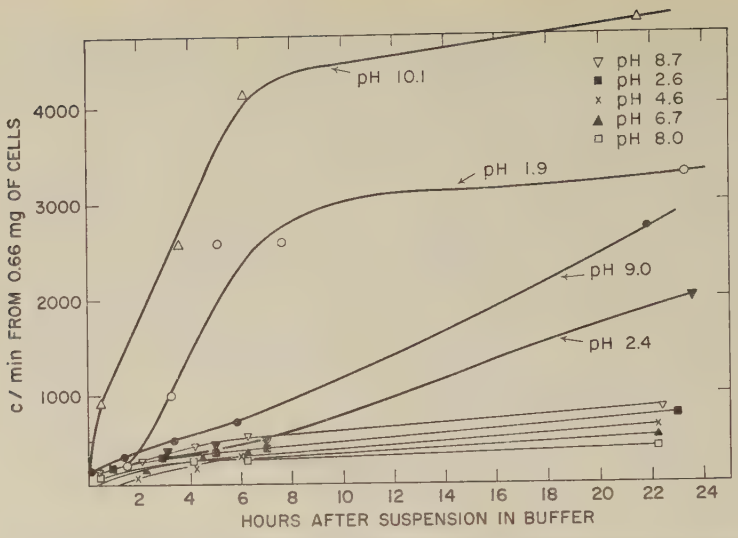

Fig. 5 Effect of phosphate buffers with different pH values on non-irradiated cells.

TABLE 1

Influence of glucose on leakage from irradiated and non-irradiated cells[1]

	Time after irradiation-hours			
	1⅓	2½	4½	6
Irradiated—glucose present	2189	3078	3509	3690
Irradiated—no glucose present	412	618	750	765
No irradiation—no glucose present	47	50	94	103
No irradiation—glucose present	98	112	152	155

[1] The data are expressed as the number of counts per minute from 0.66 mg cells. Cells in phosphate buffer, pH 7.6; irradiation time was 7½ minutes.

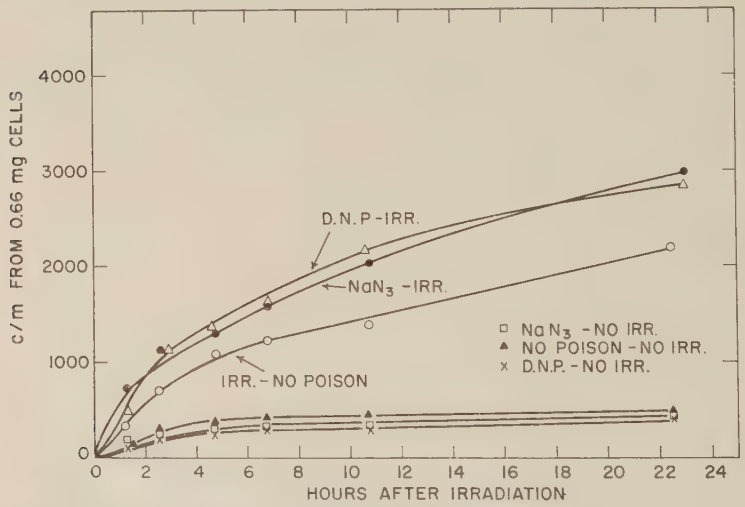

Fig. 6 Effect of 2,4-dinitrophenol (DNP) and sodium azide (NaN_3) on leakage from irradiated cells. Concentration of poisons 0.003 M.

TABLE 2

The effect of temperature on leakage of P^{32} from irradiated cells[1]

Temperature °C	Counts/min. from 0.66 mg cells leaked in 22 hrs.	Rate of leakage counts/min. from 0.66 mg cells/hr.
5	548	25
20	944	43
25	1285	57
30	1409	64

[1] Irradiation time was 7½ minutes; cells were suspended in water.

An experiment was designed to test the influence of cell metabolism on the leakage process. The experiment was carried out using a 0.05 M buffered-cell suspension at pH 6.7 because of the extra carbon dioxide produced during respiration. The final concentration of the glucose after irradiation and dilution was 0.03 M. The experiment was run for only 6 hours because after that time bacterial growth appeared in the rich medium of leakage products and glucose. The results are seen in table 1; the presence of glucose increases the loss of phosphate from irradiated cells 5-fold, but has only a slight effect on non-irradiated cells.

The influence of two metabolic poisons, 2,4-dinitrophenol, DNP, and sodium azide, NaN_3, was tested on endogenous cells. The cells were irradiated for 7½ minutes in pH 4.5 buffer and, after dilution, incubated in concentrations of 0.003 M. These poisons, it is seen in figure 6, cause leakage to increase in irradiated cells. Both influence the leakage to the same extent. Little or no effect of the poisons is seen in the non-irradiated controls.

The effect of temperature on the leakage process was tested by incubating water-suspended, 7½ minute-irradiated cells at 5°, 20°, 25°, and 30°C and following leakage for 22 hours. The time-course curves for leakage were nearly linear in all cases. The leakage rates were calculated from these curves and are presented in table 2. As expected, leakage decreases at the lower temperatures. From these data the temperature coefficient or Q_{10} for the leakage process can be calculated from the formula below:

$$\frac{\text{rate of leakage at } 30°}{\text{rate of leakage at } 20°} = \frac{64 \text{ counts/min. from 0.66 mg cells/hr}}{43 \text{ counts/min. from 0.66 mg cells/hr}} = 1.5$$

The absorption spectrum of the leakage materials from irradiated yeast cells was found to be identical with that published by Loofbourow et al. ('38). The spectrum is that of nucleic acids and their constituents with a maximum of 260 mμ. The supernatant of cells irradiated for 20 minutes and incubated for two hours read 0.82 at 260 mμ on the O.D. scale. There was no peak at 280 mμ characteristic of tyrosine, phenylalanine and the cytoplasmic proteins containing these aromatic amino acids. Water-insoluble material was present after lyophilization and addition of a small amount of water. This precipitate after washing was white and flaky. It was insoluble in ether and concentrated sodium hydroxide. After suspension in 6 N HCl for 24 hours at 100°C, followed by repeated evaporation and paper-chromatogramming, no amino acid spots were noted. To the supernatant was added an equal volume of 10% trichloracetic acid. A faint, white cloudiness appeared. Loofbourow ('42) stated that the cell-free supernatant was free of protein, but the above is interpreted as evidence that some protein is present.

The radioautograph of a paper chromatogram of the concentrated leakage material for irradiated cells is seen in figure 7. At least 15 different compounds are resolved by the solvent systems of phenol and butanol-propionic acid. Figure 8 is a radioautograph of the free phosphates in the pool obtained by extracting uniformly-labeled, well-washed cells with hot 80%, 60% and 20% ethyl alcohol. Figures 7 and 8 provide sufficient evidence that some of the leakage materials are

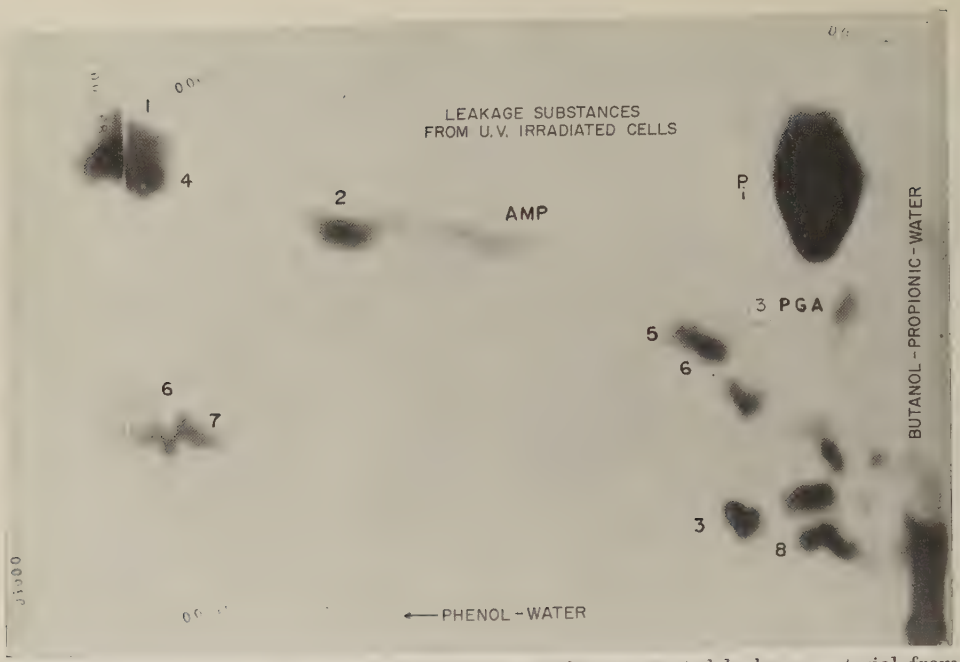

Fig. 7 Radioautograph of a paper chromatogram of concentrated leakage material from irradiated cells. See text and figure 9 for explanation of numbers and abbreviations.

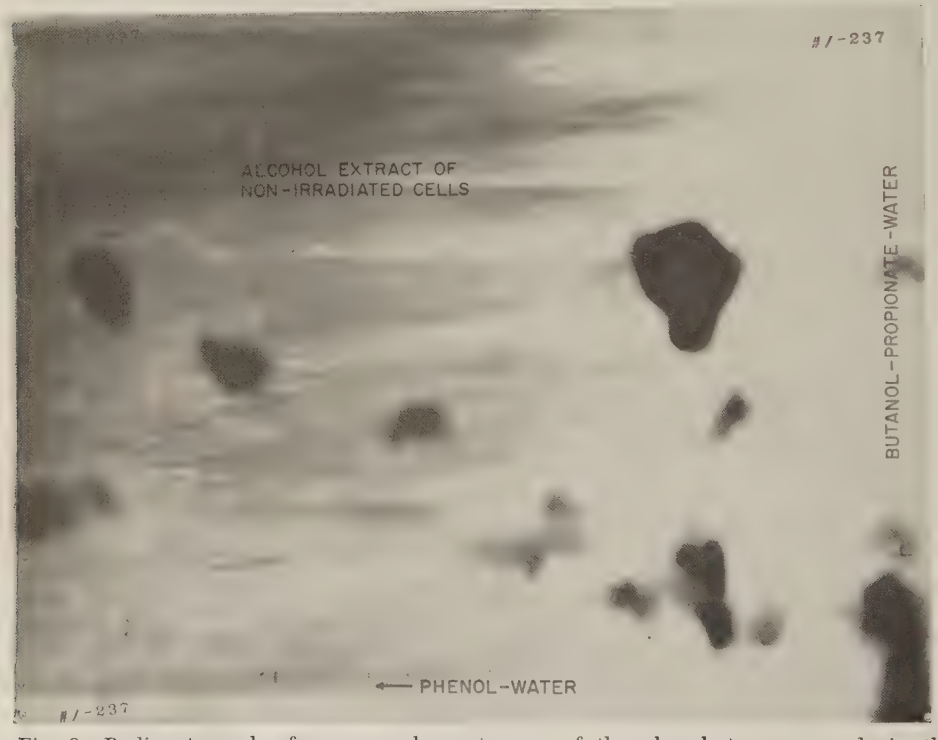

Fig. 8 Radioautograph of a paper chromatogram of the phosphate compounds in the alcohol extract of non-irradiated cells.

different from those existing free in the cell and that leakage is selective in that certain phosphates in the cell do not leak out of the cell following irradiation. Among the leakage products are inorganic orthophosphate (P_i) and 3-phosphoglyceric acid (3-PGA). These are designated on figure 7. The identification procedure involved spot elution and co-chromatography with authentic compounds followed by radioautography and spraying with acid molybdate reagent. Glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, fructose-1, 6-diphosphate, ribose-5-phosphate and flavine mononucleotide are not present. Figure 9 is another radioautograph of a chromatogram of certain radioactive leakage products. The chromatographic separation was made using solvent systems different from those employed for figures 7 and 8. The spots seen in figure 9 represent compounds which, at pH 5.5, were adsorbed on charcoal and which were subsequently eluted from the charcoal with 50% ethanol and 50% ethanol plus 1% NH_4OH. All of the mono-, di- and triphosphates of adenine, guanine, cytosine and uracil were co-chromatogramed with the charcoal-adsorbable radioactive leakage products, but the only ultraviolet-absorbing spot which coincided with a dark spot on the radioautograph was adenylic acid. All others appeared in non-radioactive areas. Uridine diphosphoglucose was also tested with negative results.

The correspondence of the spots on the chromatograms of the combinations of two solvent systems is indicated by the numbers assigned to the spots on figures 7 and 9. These relationships were worked out by cutting radioactive spots from phenol and butanol-propionic chromatograms, eluting them and co-chromatogramming them with reference compounds such as adenylic acid, uridylic acid, adenosine and adenine, using the ammonium sulfate-isopropyl alcohol, ammonium acetate-ethyl alcohol systems. Spot number 2 is

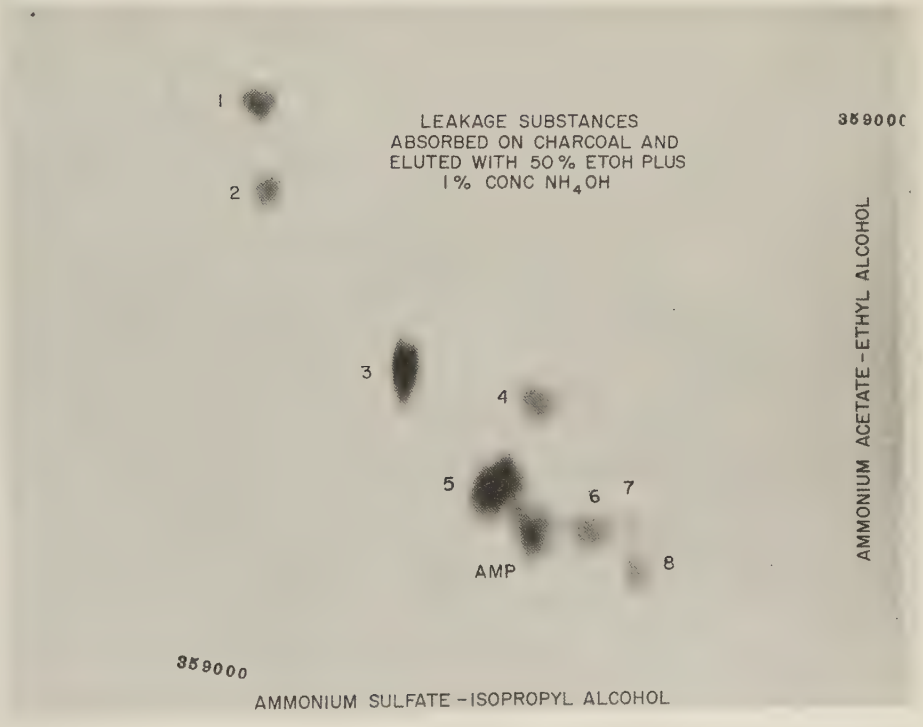

Fig. 9 Radioautograph of a paper chromatogram of leakage substances adsorbed on charcoal and eluted with 50% ethanol plus 1% concentrated NH_4OH. The numbers refer to the position of the same compound on the radioautograph shown in figure 7.

one of particular interest. It is not only an organic phosphate, as is evidenced by the radioactivity, but it is ninhydrin-positive indicating the presence of an amino group. This spot is not found on figure 8, the chromatogram of the free cellular phosphates, as evidenced by the fact that no radioactive spot in the same region colors with ninhydrin. Efforts to elute the spot, hydrolyze it and rechromatogram its hydrolytic products in order to identify the amino acid present were unsuccessful.

Amino acids also leak from the cells following irradiation (Loofbourow, '47; Swenson, '59). A detailed study of amino acid leakage will appear in a future report.

The uniqueness of the ultraviolet radiations in the matter of leakage of materials from yeast cells is illustrated by one additional experiment. The effect of elevated temperatures and subsequent irradiation was tested. Washed, 5-ml samples of uniformly-labeled cells, suspended in water, were heated for three different periods of time, 5, 10 and 20 minutes, at 55°C. Survival was approximately 0.1% after 5 minutes of heating and 0.005% after 10 minutes of heating. Another sample was heated for 20 minutes followed by washing and 20 minutes of exposure to ultraviolet light. The samples were then diluted and processed as in the previously-discussed time-course experiments. Figure 10 shows that heating the cells causes leakage and that the amount of leakage increases with the length of exposure to heat. Irradiation of cells subsequent to heating causes additional leakage. The chromatograms of the leakage products from cells heated and then incubated for three hours (fig. 11), and from cells heated, washed, irradiated and incubated for three hours (fig. 12) show striking differences. The leakage materials resulting from heating alone are primarily inorganic orthophosphate; the heated-irradiated products are, with minor differences, the same as obtained by irradiation alone.

DISCUSSION

The term "leakage" has been used in this paper to describe the movement, from the inside of the cells to the extracellular medium, of substances normally restrained in their movement. This term describes an overall process and implies nothing concerning the mechanism, in much the same manner that the term "permeability" denotes the movement of a substance from the external medium to the interior of a cell, without specifying whether the movement is by diffusion or by means of an active-transport system. Until the process is more completely understood it seems fair and convenient to speak of the leakage of materials from irradiated yeast cells.

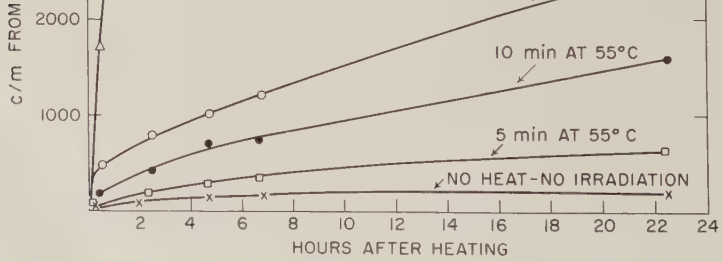

Fig. 10 The effect of high temperature and of irradiation following exposure to high temperature on leakage.

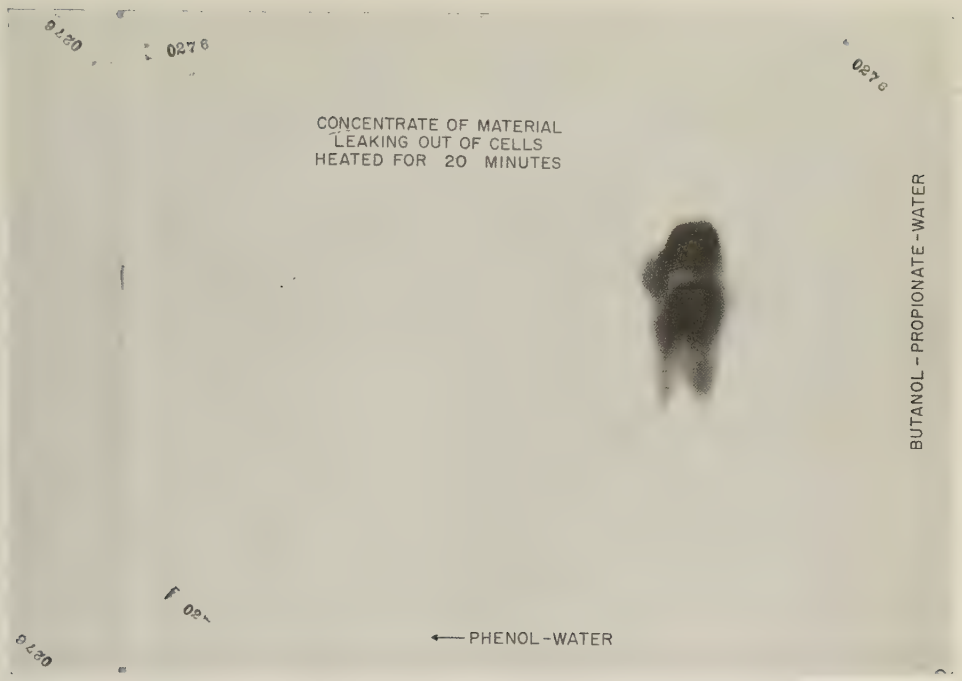

Fig. 11 Radioautograph of a paper chromatogram of the P^{32}-containing leakage substances of cells exposed to high temperature.

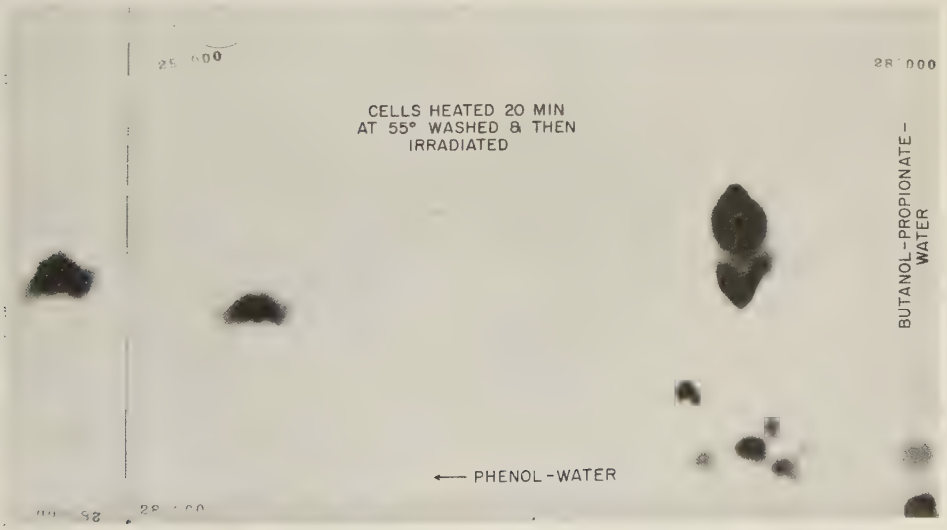

Fig. 12 Radioautograph of a paper chromatogram of irradiated cells which had been previously exposed to high temperature.

The emphasis of Loofbourow and his associates ('38, '41, '47) was on what the authors termed proliferation-promoting intercellular hormones that were released (leaked) from the cells following irradiation with ultraviolet light. The assay procedure involved measurement of growth of non-irradiated yeast to which concentrated

leakage materials were added. This required irradiation of large volumes of yeast as well as long irradiation and incubation times. The present experiments include time-course studies which indicate that the amount of leakage varies with a number of factors including the irradiation dosage and the time allowed to elapse after irradiation. Following irradiation with low dosages, 5 minutes or 7½ minutes of exposure, leakage is continuous and at a steady rate for at least 24 hours. At high dosages (20 minutes or more exposure), leakage is rapid and within 4 hours the maximum seems to be attained.

Loofbourow et al. ('41) varied the irradiation time required to kill 90% of the cells (as indicated by methylene blue staining) and found that the active factors causing cell proliferation appear before cell death. A different criterion of death—the ability to form colonies—was employed in the present experiment; however, the data show that very few cells survive the treatment required to cause leakage. Cell death, however, does not mean that all cell functions are stopped; indeed, the overall rate of respiration is but little affected by lethal dosages of ultraviolet light (Swenson, '58). The synthetic functions of the cell are probably the most sensitive to these radiations.

It is not likely that much of the P^{32} activity in the medium attributed to leakage is caused by the disintegration of cells. Half of the total number of P^{32} counts of non-injured cells may appear in the medium of irradiated cells, but the total number of cells remains constant. The selective nature of the leakage is also an argument against cell distintegration; the chromatograms show clearly that some free cellular constituents do not appear among the leakage products. The presence of a small amount of protein is difficult to explain on a basis other than cell disintegration, but the passage of proteins across intact cell membranes is not unknown. The cell membrane is a selective barrier between the cell interior and the extracellular medium. Even when there is an unfavorable gradient across the membrane, nutrients, ions, and other substances are accumulated by the cell. The retention of these materials probably requires energy, but in the absence of an exogenous source such as glucose the endogenous rate of respiration is quite low. It is, therefore, remarkable that yeast cells uniformly labeled with P^{32} and suspended in water lose only about 1% of their labeling in a 24-hour period.

A possibility that comes immediately to mind to explain the leakage phenomenon is that the cell membrane is damaged, permitting cellular constituents to diffuse from the inside to the outside of the cell. It was hoped that the temperature coefficient experiments would provide evidence for or against this possibility. The Q_{10} value characteristic of a thermochemical process between 2 and 3, while that of a diffusion process is considerably less than 2, but greater than 1 (see Giese, '57). The value of 1.5 which was obtained for the interval 20° to 30°C is consistent with a process in which membrane damage occurs, allowing small particles to diffuse outward. If this were so, radioactive orthophosphate on the ouside should enter faster than in non-irradiated cells; in other words, the permeability should increase. This has not been found to be true; ultraviolet radiations decrease the permeability of the cell to orthophosphate (Swenson, '58). Loofbourow ('42) conducted action spectrum studies on the leakage process and found maximal spectral efficiency in the region of 265 mμ. This indicates that the photolabile constituent may be nucleic acids. The action spectrum for the inhibition of induced-enzyme formation by ultraviolet light is also in the region of 260 mμ (Swenson, '50). Nucleic acids are known to play important roles in protein synthesis; quite possibly the ultraviolet radiations are absorbed by nucleic acids and, in some way, energy-requiring processes such as synthesis and active transport are inhibited. Under these conditions molecules that diffuse out of the cell would not be transported back in.

The influence of metabolism on the leakage process is partially consistent with reasoning just outlined. With metabolic cycles operating rapidly but with synthesis limited or not taking place, inorganic phosphate from degraded organic phosphates would enter the phosphate pool and diffuse to the exterior. However, this does no

ake into account the large amount of rganic phosphates which also leak out of he cell.

The poison experiments are difficult to xplain in the light of the previous discus- ion. Sodium azide and 2,4-dinitrophenol re both uncoupling agents (Loomis and ipmann, '49) which prevent the forma- ion of ATP and are, therefore, inhibitors f energy-requiring activities in cells. It vould be expected that in non-irradiated ells these poisons would cause leakage of hosphate to occur. The fact that they do ot is taken as evidence that ultraviolet adiations have some unique and specific ction in causing leakage from irradiated ells; their uncoupling action may make nore phosphate available to the pool be- ause it does not become incorporated into TP.

Billen ('57) has reported that in ultra- iolet and x-irradiated cells of *Escherichia oli* the loss of cellular constituents, in- luding nucleic acid fragments, is inhib- ted by the absence of an exogenous energy ource, the presence of arsenate and low emperature of incubation. The loss is also ependent on the presence of orthophos- hate in the medium. The present experi- nents on yeast indicate that the pH of the hosphate buffer serving as the suspending nedium is of more importance than the resence or absence of phosphate. Both he rate and extent of leakage are affected y the external pH. Information is lacking n the influence of external pH on the pH f the interior of irradiated cells, but nor- nally the pH of the cytoplasm is relatively ndependent of the external medium. It eems reasonable to conclude that the in- luence of pH is on the cell membrane. Within the pH range 2.6 to 8.7, cellular onstituents are retained within the cell y the expenditure of energy from endog- nous metabolic reactions. It is postu- ated that following irradiation energy is o longer available for retention of these ubstances and that some modifications ccur in the cell membrane. These modifi- ations may be caused by changes in the onfiguration of membrane proteins at dif- erent pH values, thus, allowing different egrees of leakage. Membrane modifica- on is also indicated in the experiments here leakage occurs to varying extents in non-irradiated cells when the pH of the suspending medium is below 2.6 and above 8.7.

The specific nature of ultraviolet radiations on the production of leak- age materials is well illustrated in the high-temperature experiments. Ex- posure of cell suspensions to 55°C for 20 minutes killed more than 99.9% of the cells but leakage, while con- siderable, did not approach that attained by irradiated cells. Furthermore, dead cells, whose proteins were probably de- natured at this temperature, were sensitive to ultraviolet radiations and leaked con- siderable amounts of radioactive material to the medium. Energy relations would be of little importance here. The evidence points to at least two possibilities, mem- brane damage and breakdown of large compounds within the cell. The first pos- sibility is the more likely, because a com- plex of materials are in the cell but ortho- phosphate is the primary leakage product from heated cells. The second possibility may play a role in the extent of leakage from irradiated cells.

There seems to be no question concern- ing the fact that ultraviolet radiations are unique agents in causing materials to leak from yeast cells. The chromatogram work provides good evidence that certain sub- stances found free in the cell do not leak out following irradiation. In other words, leakage is selective. Of more importance is the appearance among the leakage pro- ducts of substances which were not among the free constituents of the cells. Loof- bourow et al. ('47) reported that spectro- scopic evidence indicated synthesis of materials absorbing at 260 mμ as a result of stimulation by ultraviolet light. The stimulation of synthesis seems unlikely in view of what is known about the damage done by these radiations but there is not enough evidence to dismiss the possibility. It is also possible that degradation of larger molecules such as nucleic acids might oc- cur. Such a hypothesis also presents diffi- culties primarily because only one common nucleotide, adenylic acid, is found on the chromatogram of the leakage products. This is perplexing because Schmitz ('54) has reported the presence of many free nucleotides in yeast cells including the

mono, di and tri forms of the 4 nucleotides of ribonucleic acid. The present results are also not in harmony with those of Thomas, Hershey, Abbate and Loofbourow ('52) who determined the nucleotides in the suspending medium from irradiated yeast by ion-exchange chromatography. They reported the presence of cytidylic, adenylic, uridylic and guanylic acids as well as polynucleotides of RNA which yielded the 4 acids on hydrolysis. It is of passing interest that none of the 260 mμ absorbing fractions which came off the column had growth-stimulating activity.

The problem of growth-stimulating factors is an important one, but also of great interest is the problem of the mechanism of the action of ultraviolet radiations on the cell in causing the leakage. Most ultraviolet studies have been concerned with lethal and mutagenic effects. The present study of the leakage phenomenon, in which unique substances appear in the suspending medium following radiation damage, indicates that this system may provide an opportunity to study radiation damage to cells in terms of specific chemical reactions.

SUMMARY

1. A study was made of the action of ultraviolet radiations on the leakage of radioactive phosphate, P^{32}, from uniformly-labeled yeast cells.

2. At low dosages leakage is slow and steady for a 24-hour period; at high dosages leakage is rapid and the maximum amount occurs within 4 hours after irradiation. At high dosages 50% of labeled phosphate of the cells is found in the medium after 4 hours.

3. The pH of the medium influences leakage caused by ultraviolet light. Minimum leakage occurs in the region of pH 6. Above pH 8.7 and below pH 2.6 leakage occurs to a considerable extent in non-irradiated cells.

4. Sodium azide and 2,4-dinitrophenol cause increased leakage in irradiated cells, but these poisons do not cause leakage from non-irradiated cells.

5. Leakage from irradiated cells metabolizing glucose is 5 times that from irradiated cells having no exogenous substrate.

6. The temperature coefficient or Q_{10} for leakage caused by irradiation is 1.5 for the interval 20° to 30°C.

7. A paper chromatographic analysis was made of the leakage products. Positive identification was made of inorganic orthophosphate, 3-phosphoglyceric acid and adenylic acid. Unique substances appear to be present among the leakage products that are not present in the cells.

8. Leakage is selective. Certain substances which are present in the cell do not leak out following irradiation.

9. The leakage substances resulting from ultraviolet irradiation differ from those resulting from heating the cells at 55°C. Cells rendered non-viable by heating leak a considerable additional amount of phosphate which is chromatographically similar to that from cells irradiated but not heated.

10. The possible causes of leakage of phosphate from irradiated cells are discussed. The process is not a simple one but the evidence points to membrane damage allowing diffusion of cellular constituents to the exterior as well as to interference with energy-coupled processes in the cell.

ACKNOWLEDGMENT

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Histochemical Localization of Some Enzymes in the Kidney of a Hibernator[1]

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That hypothermia exerts a profound influence upon renal physiology has been amply demonstrated (Bickford and Winton, '37; Page, '55; Segar, Riley, and Barila, '56; Hernandez and Coulson, '57) but little information is available pertaining to kidney function during hibernation (Hong, '57). Upon reduction of body temperature there is a decrease in renal blood flow and a depression of both tubular reabsorption and secretion (Hong and Boylan, '59). Certain metabolic transport mechanisms which are cold sensitive as a consequence of their high energy requirements are inhibited during hypothermia (Segar, '58).

During hibernation in the ground squirrel the body temperature and the metabolic demands of the organs are greatly reduced (Zimny and Gregory, '58). Urinary excretion has ceased and upon sacrificing, the urinary bladder is found distended. Studies of the histochemical localization of various enzymes in the kidneys of the rat and man (Sternberg, Farber and Dunlap, '56; Nachlas, Walker and Seligman, '58; Wachstein, '55) have added greatly to the knowledge of renal physiology. The purpose of this paper is to demonstrate the localization of some enzymes in a hibernator so as to aid in the interpretation of kidney function during hibernation.

MATERIALS AND METHODS

Kidneys were obtained from 30 thirteen-lined ground squirrels, *Citellus tridecemlineatus*, 15 experimentals that had hibernated for at least a week at 3–5°C and 15 controls housed at an environmental temperature of 25–27°C. The control animals were sacrificed under nembutal anesthesia, the hibernators were sacrificed while hibernating and both kidneys removed immediately. One kidney was fixed in 10% formalin. The other kidney was cut down the longitudinal axis and one-half immediately frozen on the stage of the freezing microtome. Sections 10 μ in thickness were mounted directly on coverslips, allowed to dry for about one minute at room temperature (25–27°C) flooded with the following media and then placed in a constant temperature oven (37°C).

For Succinic Dehydrogenase	
	ml
0.1 M phosphate buffer, pH 7.4	1.0
0.5 M sodium succinate, pH 7.4	0.3
Nitro blue tetrazolium (NBT) (1 mg/ml)	0.7
H_2O to	3.0

For DPN Diaphorase	
	ml
0.2 M phosphate buffer, pH 7.4	1.0
DPNH (reduced DPN) 10 mg/ml	0.3
Nitro blue tetrazolium (NBT) (5 gm/ml)	0.3
H_2O to	3.0

For TPN Diaphorase	
	ml
0.1 M veronal buffer, pH 7.4	0.9
TPNH (reduced TPN) 10 mg/ml	0.3
Nitro blue tetrazolium (NBT) (5 mg/ml	0.3
H_2O to	3.0

The sections were allowed to incubate until maximum color had developed without visible precipitation in the media, then rinsed in 1% glacial acetic acid followed by buffered formalin, and finally water. After drying in the air, they were mounted in glycerine jelly (Seligman and Rutenburg, '51; Farber, Sternberg and Dunlap, '56).

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[2] Trainee Fellow, National Heart Institute, HTS-5111(C1).

For glucose-6-phosphate the following procedure was used: The mounted sections were incubated in:

1. Potassium glucose-6-phosphate—2.0 ml of 125 mg%;
2. Tris (hydroxy methyl) aminomethane malate (tris malate) pH 6.7—2.0 ml of 0.2 M;
3. lead nitrate—0.3 ml of 2%;
4. distilled H_2O—0.7 ml.

The sections were then washed in distilled water; immersed in 2% yellow ammonium sulfide for two minutes; fixed in 10% formalin and mounted in glycerine jelly (Chiquoine, '52).

For alkaline phosphatase the half of the kidney that was fixed in 10% formalin, was sectioned at 10 μ and the sections mounted on uncoated slides. The slides were incubated at 37°C for 20 minutes according to the method of Gomori ('52).

Twenty-four hour urine samples were collected from several control animals by means of a metabolic cage and the water intake recorded during this period. Urine samples from hibernating animals were taken by means of bladder puncture at the time of sacrifice. The following determinations were made on the urine samples (fig. 1): glucose by the Nelson-Somogyi method ('44); sodium and potassium by flame photometry; chlorides after the method described by Schales and Schales ('41); and urea by the Conway diffusion method (Conway and Byrne, '33).

RESULTS

Diphosphopyridine nucleotide diaphorase (DPND). The proximal and distal convoluted tubules in the outer cortex gave a good reaction and the arched collecting tubules were moderately stained (fig. 3). The proximal straight tubules of the inner renal cortex gave the most intense reaction for DPND (fig. 4). In the outer medulla the intensity of the staining reaction decreased in the following order: thin loops, collecting tubules and thick ascending limbs. The collecting tubules in the papilla stained well (fig. 5). No differences were noted between the hibernated and control groups.

Succinic dehydrogenase. Succinic dehydrogenase activity was greatest in the outer zone of the renal cortex (fig. 6). The proximal convoluted tubules were stained intensely (fig. 7). In the outer medulla the distal convoluted tubules and medullary rays gave a reddish-purple reaction (fig. 8). The thick ascending loops stained well, the collecting ducts stained poorly and the thin loops were not stained. The papilla showed very little to no staining in the collecting ducts. In kidneys from hi-

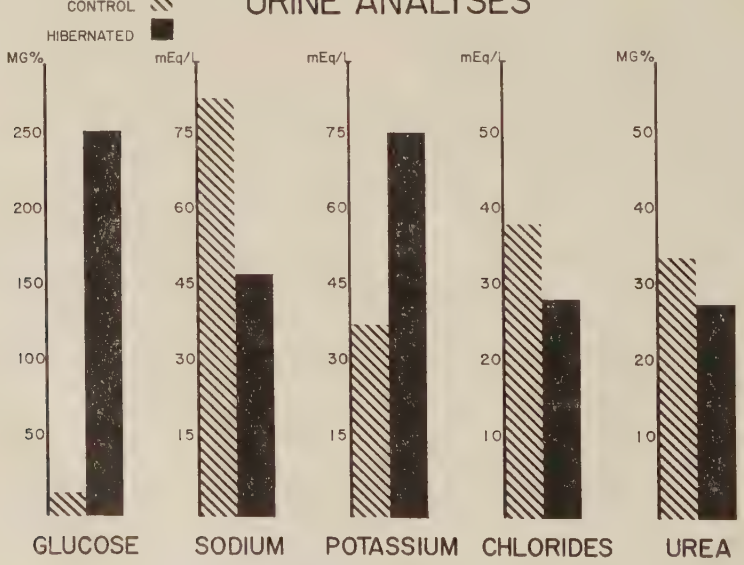

Figure 1

bernated animals an increased reaction was noted in the outer medulla (fig. 9).

Triphosphopyridine nucleotide diaphorase (TPND). Maximal intensity was in the cortex (fig. 10) with the proximal convoluted tubules staining intensely (fig. 11). In the outer medulla the thick ascending loops gave a moderate reaction, the collecting ducts a weak reaction and the thin loops were not stained (fig. 12). The collecting ducts in the papilla gave a slight reaction (fig. 13). No differences between the hibernated and control groups were demonstrable.

Glucose-6-phosphatase. Glucose-6-phosphatase activity was confined to the proximal tubules (fig. 14). The entire cell was stained with this reaction. No difference was noted between the hibernated and control animals.

Alkaline phosphatase. Alkaline phosphatase activity was confined to the renal cortex. Maximal staining occurred in the brush borders of the proximal straight tubules and the brush border of the proximal convoluted tubules (fig. 15). The other tubules and glomeruli were not stained. The kidneys of the hibernators showed the same distribution and intensity as did the controls.

A summary of these staining reactions for the control animals is given in figure 2. These data also apply to the hibernated group with the exception of the increased reaction noted in the outer medulla in sections stained for succinic dehydrogenase.

Sections stained with Mallory-azan and hematoxylin and eosin showed that kidneys from the hibernated group had markedly congested vasae rectae (figs. 18 and 19) and glomeruli filling most of the capsular space (fig. 21). Kidneys from the control group had vasae rectae that were almost bloodless and indistinct (figs. 16 and 17) and the glomeruli did not appear congested and enlarged (fig. 20).

The water intake of the control animals averaged 34 ml per 24 hr. and the urinary output averaged 15 ml. The values for the hibernating animals were zero in both cases. As shown in figure 1 significant changes in urine composition occurred during hibernation as follows: glucose increased 1575% ($P < 0.01$); potassium increased 100% ($P < 0.01$); and sodium decreased 42% ($P < 0.01$). Although chloride and urea decreased 26% and 18%, respectively, the changes are not significant.

DISCUSSION

In accordance with past studies showing the maintenance of the biochemical levels of high-energy phosphates in various tissues during hibernation in the ground squirrel (Zimny and Gregory, '58) the present study shows a maintenance of enzyme localization in the kidney. The localization of alkaline phosphatase and

LOCALIZATION OF ENZYMES

CONTROL ANIMALS

	ALK-PO4	G-6-P	SD	DPND	TPND
GLOMERULI	0	0	0	±	0 to ±
PROXIMAL CONVOL TUBULES	+++	+++	+++	+++	++
PROXIMAL STRAIGHT TUBULES	++++	+++	++	++++	+++
THIN LOOPS	0	0	0	++	±
THICK LOOPS	0	0	++	++	++
DISTAL CONVOL TUBULES	0	0	+++	+++	++
COLLECTING DUCTS					
OUTER MEDULLA	0	0	++	++to+++	+
PAPILLA	0	0	±	+++	±

Figure 2

glucose-6-phosphatase exclusively in the proximal tubule lends support to the phosphorylation-dephosphorylation theory of glucose reabsorption (Drabkin, '48; Wilmer, '44). If it is assumed that sites of high DPND, TPND and succinic dehydrogenase activity indicate areas of maximal aerobic metabolism (Lowell, Greenspon, Krakower and Bain, '53) then it seems that the proximal tubule is the most metabolically active portion of the nephron.

The highly significant increase in urinary glucose during hibernation agrees with the hypothermic studies of (Hong, '57; and Hernandez and Coulson, '57) who speculated that the enzymes necessary for glucose reabsorption were inhibited by cold and no tubular reabsorption of glucose occurred. Lyman ('55) showed that during hibernation in the ground squirrel blood glucose decreases to approximately two-thirds of the control level which would also support the idea of decreased glucose reabsorption.

During hibernation there is no water intake and no perceptible urinary excretion. Hemoconcentration occurs along with reduced peripheral blood flow and blood volume (Svihla and Bowman, '52). Urinary NaCl decreases one-fiftieth in the marmot during hibernation and a decrease in serum potassium occurs (Kayser, '53). The vascular congestion noted in the glomeruli and vasae rectae of the hibernating kidney agree with the reports of reduced renal blood during hypothermia (Bickford and Winton, '37; Hong, '57). It seems logical that the kidney during hibernation must continue to reabsorb and conserve water. The decreased urinary sodium and chloride levels during hibernation are consistent with a continued or increased water reabsorption. It is believed that this continued reabsorption of water and sodium combined with a markedly decreased renal blood flow is responsible for the cessation of urinary output during hibernation. For purposes of maintaining osmotic balance in the urine during these adjustments the kidney apparently substitutes potassium for sodium.

During hibernation in the ground squirrel enzyme activity may be depressed but the activity sites are in general maintained. Kidney physiology is altered in terms of vaso-congestion and water conservation.

SUMMARY

1. Alkaline phosphatase, glucose-6-phosphatase, succinic dehydrogenase and DPN and TPN diaphorases were histochemically localized in the kidneys of hibernated and control ground squirrels.

2. The distribution of these enzymes was the same in both groups except for the intensity of the succinic dehydrogenase staining reaction in the hibernated animals.

3. Vaso-congestion of the glomeruli and vasae rectae was pronounced in the hibernated group.

4. Significant increases in urinary glucose and potassium and a significant decrease in sodium took place during hibernation.

5. During hibernation the sites of enzyme activity were maintained and renal adjustments made in favor of water conservation.

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PLATE 1

EXPLANATION OF FIGURES

3 Fresh frozen section from the kidney of a control ground squirrel stained for DPN Diaphorase showing a good reaction in the proximal (P) and distal (D) convoluted tubules. × 24.

4 Fresh frozen section from the kidney of a control ground squirrel stained for DPN Diaphorase showing a good reaction in the proximal (P) convoluted tubules. × 97.

5 Fresh frozen section from the kidney of a control ground squirrel stained for DPN Diaphorase showing the collecting tubules (CT) in the papilla well stained. × 24.

6 Fresh frozen section from the kidney of a control ground squirrel stained for succinic dehydrogenase showing the outer cortex well stained. × 24.

7 Fresh frozen section from the kidney of a control ground squirrel stained for succinic dehydrogenase showing the proximal (P) convoluted tubules in the cortex stained intensely. × 390.

8 Fresh frozen section from the kidney of a control ground squirrel stained for succinic dehydrogenase showing a slight reaction in the outer medulla (OM). × 24.

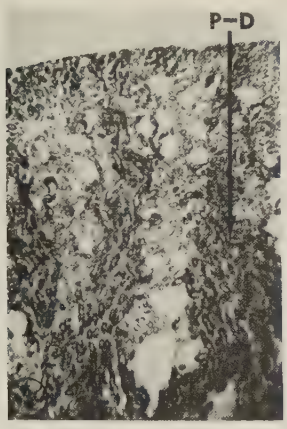
9 Fresh frozen section from the kidney of a hibernated ground squirrel stained for succinic dehydrogenase showing an increased staining reaction in the outer medulla (OM). × 24.

10 Fresh frozen section from the kidney of a control ground squirrel stained for TPN Diaphorase showing maximal staining in the cortex. × 24.

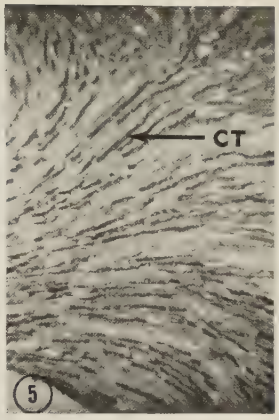
11 Fresh frozen section from the kidney of a control ground squirrel stained for TPN Diaphorase showing intense staining of the proximal (P) convoluted tubules. × 390.

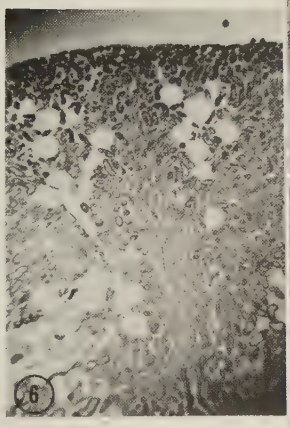
12 Fresh frozen section from the kidney of a control ground squirrel stained for TPN Diaphorase showing the contrast in staining reaction between inner cortex (IC) and outer medulla (OM). × 24.

13 Fresh frozen section from the kidney of a control ground squirrel stained for TPN Diaphorase showing the slight reaction in the papilla (PAP). × 24.


P–D


P
4


CT
5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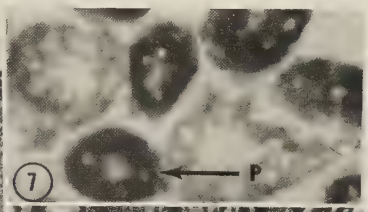
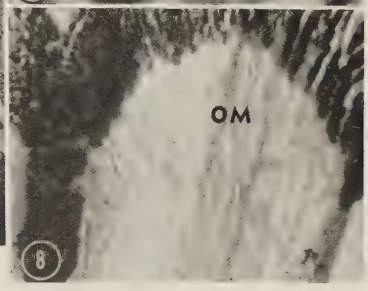
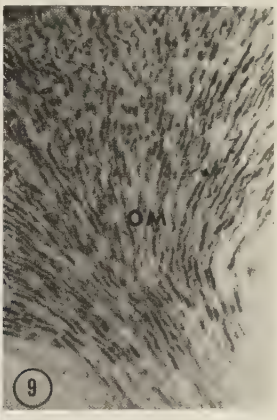

7
P


OM
8


OM
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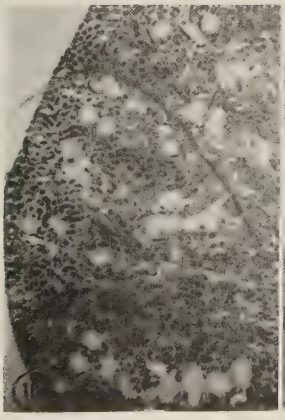
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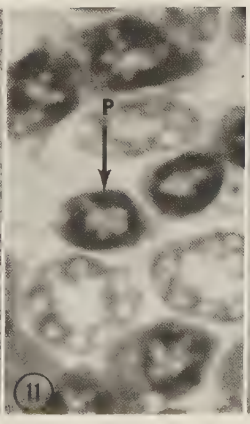

P
11


OM
IC
12

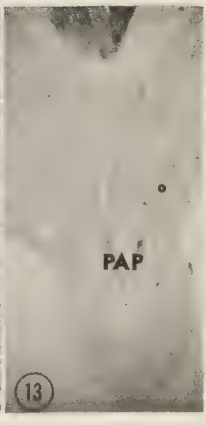

PAP
13

PLATE 2

EXPLANATION OF FIGURES

14 Fresh frozen section from the kidney of a control ground squirrel stained for glucose-6-phosphatase showing activity in the proximal (P) tubules. × 27.

15 Fresh frozen section from the kidney of a control ground squirrel stained for alkaline phosphatase showing maximal staining in the proximal (P) tubules. × 27.

16 Paraffin section from the kidney of a control ground squirrel stained with Mallory-azan showing bloodless vasae rectae (VR). × 27.

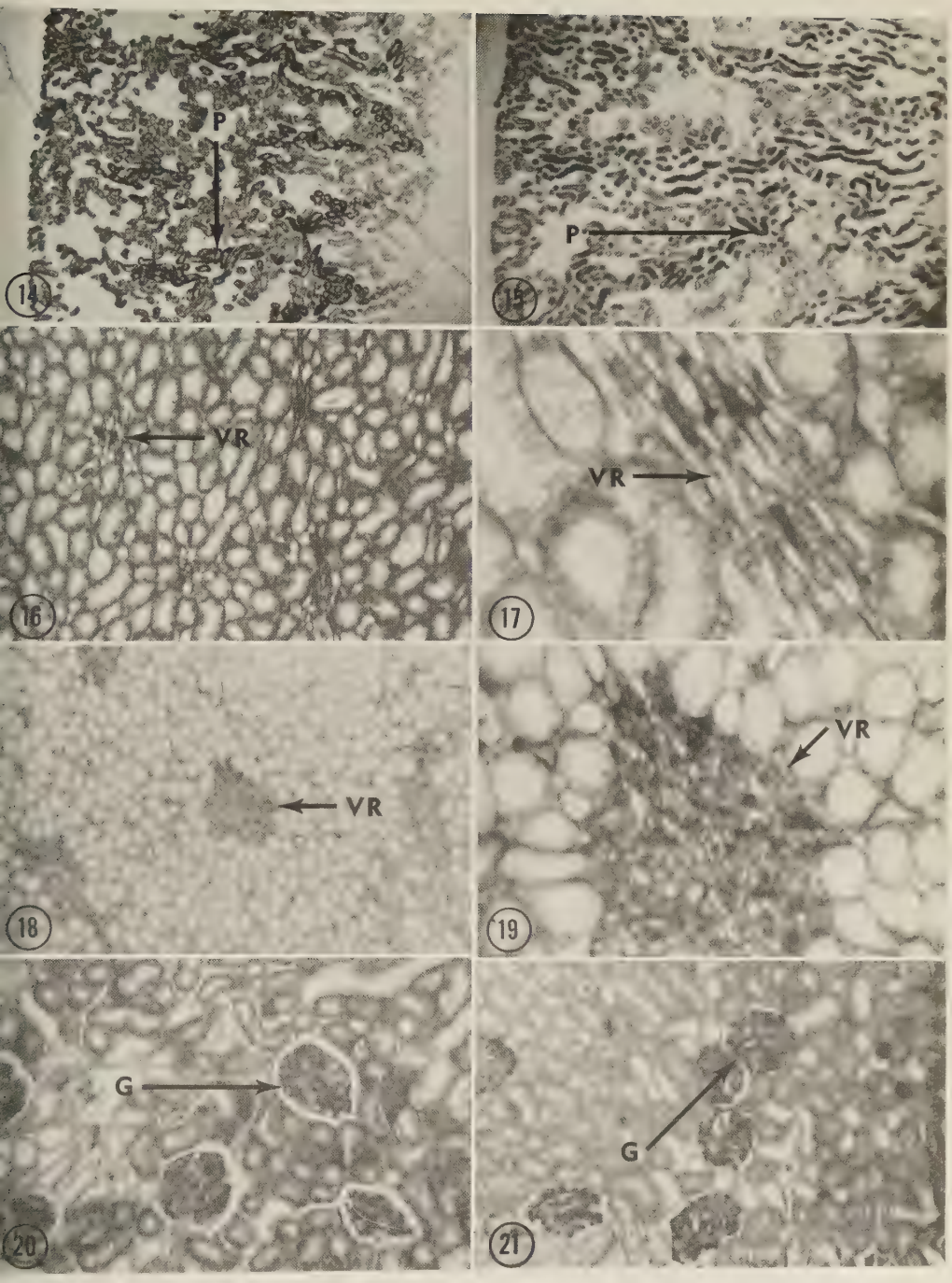
17 Paraffin section from the kidney of a control ground squirrel stained with Mallory-azan showing a bloodless vasa recta (VR). × 440.

18 Paraffin section from the kidney of a hibernated ground squirrel stained with Mallory-azan showing congested vasae rectae (VR). × 27.

19 Paraffin section from the kidney of a hibernated ground squirrel stained with Mallory-azan showing a congested vasa recta (VR). × 440.

20 Paraffin section from the kidney of a control ground squirrel stained with Mallory-azan showing non-congested glomeruli (G). × 110.

21 Paraffin section from the kidney of a hibernated ground squirrel stained with Mallory-azan showing congested glomeruli (G). × 110.


P
14
P
15
VR
16
VR
17
VR
18
VR
19
G
20
G
21

Monosaccharide Penetration into Human Red Blood Cells by an Altered Diffusion Mechanism[1]

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In 1911, Rona and Döblin first demonstrated that glucose penetrated into human red blood cells. Since then, the mechanism by which monosaccharides enter the human red cell has concerned many investigators.

Masing ('14) noted that the permeability for glucose was markedly inhibited by lowering the temperature. Glucose reached equilibrium 50 times faster at 25°C than at 0°C. He concluded that this high temperature coefficient was an indication that free diffusion to equilibrium was not taking place and that perhaps a chemical reaction was involved in the penetration of this sugar. It was then postulated that if glucose reacted chemically while penetrating the red cell, then perhaps it was penetrating against a concentration gradient. Investigations were conducted to determine whether or not naturally occurring or added glucose in the blood becomes equally distributed between the environmental medium and the red cells. Ege, Gotlieb and Lakestraw ('25), Ege and Hansen ('27) and Andreen-Svedberg ('33) definitely established that the blood glucose in man is evenly distributed between the water of the plasma and the corpuscles.

Kozawa ('14), who worked with ⅔ isotonic sugar solutions (containing little or no electrolytes) at room temperature, showed by the use of the hematocrit method and direct chemical analysis, that pentoses penetrated faster than hexoses. However, he indicated that the time to equilibrium was not the same for all the hexoses. Glucose and fructose reached equilibrium more slowly than galactose, mannose and sorbose and the fastest to reach equilibrium were the pentoses, arabinose and xylose. Fleischmann ('28) reported similar results. Wilbrandt ('38) claimed that the relative rates of penetration of various sugars (in decreasing order) were as follows: xylose, arabinose > mannose > galactose > glucose > sorbose > fructose. Further evidence demonstrating the high degree of stereochemical specificity to monosaccharides by the human red cell has been noted by Wilbrandt ('47), who showed that D-xylose and L-arabinose entered the red cell fairly fast, whereas L-xylose and D-arabinose were essentially unable to penetrate.

The undisputed facts known so far concerning the penetration of monosaccharides into the human red blood cell are: a high temperature coefficient accompanies sugar penetration, sugars always penetrate to a diffusion equilibrium and never against a concentration gradient and the relative rates of monosaccharide penetration are dependent upon steric and structural differences among the sugars.

Four different mechanisms have been postulated to explain the penetration of sugars into the human red blood cell. These mechanisms are free diffusion, active transport, facilitated diffusion (a modification of active transport), and altered diffusion, the mechanism postulated in this paper.

If monosaccharides enter the human red cell by free diffusion, then the movement of the sugar molecules must take place under the driving force of thermal agitation, unrestricted by steric factors and un-

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limited by the molecular structure of the environment (Danielli, '54). Penetration of monosaccharides to a diffusion equilibrium and no penentration against a concentration gradient are data in support of this hypothesized mechanism. Also Mawe ('56) has shown that, at 37°C and pH 7.78, glucose penetration into the human red cell obeys Fick's law of diffusion, which means that the rate of penetration of glucose is a linear function of the difference in concentration of glucose on both sides of the red cell membrane.

Other mechanisms have been postulated for sugar penetration because this phenomenon is accompanied by a high temperature coefficient and variations in the relative rates of sugar penetration can be associated with structural and stereochemical differences among the monosaccharides. Danielli ('54) defines active transport as the transferring of molecules across a membrane by the use of an energy supply other than, or additional to thermal agitation. There are two schools of thought concerning active transport of sugars into the human red cell.

Wilbrandt and Rosenberg ('51) believe that monosaccharides penetrate by an enzymatically controlled active transport mechanism. They hypothesize that this is accomplished by the combined action of two surface enzymes separated by the cell membrane which temporarily change the monosaccharide to a membrane soluble transport form capable of penetrating the membrane and thereby enabling the sugar to enter the cell. Facilitated diffusion has been proposed by LeFevre and LeFevre ('52) and is a modification of this enzymatically controlled active transport mechanism. They suggest that sugars form a highly dissociated complex with some factor in the red cell surface (a "carrier") and this "carrier" transports the sugar to the interior of the cell. The LeFevre hypothesis allows the "carrier" to effectively operate in both directions across the cell membrane, whereas in the scheme suggested by Wilbrandt and Rosenberg, the individual sugar complex operates in only one direction and necessitates two enzymes, one which couples with the sugar molecule at the outer surface of the membrane, and another which liberates the sugar molecule at the inner surface of the membrane.

The altered diffusion mechanism

Since neither of the previously mentioned mechanisms for sugar penetration into human red cells completely explain this phenomenon, another mechanism will be postulated. This mechanism will be called the altered diffusion mechanism. Penetration of sugars into the human red cell by such a mechanism simply means that sugars are entering the red cell to a diffusion equilibrium under the driving force of thermal agitation. However, their ability to diffuse into the cell is regulated by the state or condition of the cell membrane and the structural configuration of the sugar molecule. In other words, the relationship between the sugar molecule and the size, charge and hydrogen bonding (Parpart and Ballentine, '52; Danielli, '54) of the aqueous channels in the pores of the membrane are determining factors in the rate of sugar penetration into the human red cell.

The aqueous channels in the plasma membrane may be spiral or convoluted in shape. By virtue of this phenomenon, a partial barrier to diffusion is established. Consequently, a high temperature coefficient would be associated with sugar penetration because this resistance to diffusion is overcome directly by thermal agitation (Danielli and Davson, '34). The shape of these aqueous channels may also account for the highly selective properties of the plasma membrane for monosaccharides.

The altered diffusion mechanism would eliminate the necessity of postulating a "carrier" or an enzyme transport for sugar penetration into the human red cell.

The pupose of this investigation is to test the hypothesis that monosaccharides enter the human red cell by the altered diffusion mechanism. In order to correlate some of the pertinent data in the literature with this hypothesis, the effects produced by varying the pH upon the relative rate of penetration of hexoses and pentoses must first be studied. It is possible that much of the confusion in the literature associated with sugar penetration into the human red cell has arisen because pH was not controlled in many experiments

n many instances, even when a buffered H was used, the pH has been changed rom experiment to experiment or even uring an experiment. The temperature oefficients for sugar penetration will also e investigated in relation to changes in H.

The claim that high glucose concentra-ons (greater than the isosmotic con-entration of 0.3 M) inhibit the rate of enetration of this sugar will be studied at arious pH's. Also the effect of various ower concentrations—in the range from .006 M (approximately 100 mg %) to .3 M of glucose—on the rate of glucose enetration at various pH's will be investi-ated.

To establish that a relationship exists etween the cell membrane and the pene-rating monosaccharide, the structural pecificity as well as the optical rotation of ne sugars will be examined in the light of neir relative rates of penetration. The ates of penetration of the α- and β-isomers f D-glucose will be investigated to estab-sh whether or not they penetrate at the ame rate. Finally, an attempt will be nade to discern whether or not hydrogen onding is an important factor in the pene-ration of glucose into the human red lood cell.

MATERIALS AND METHODS

The relative rates of monosaccharide enetration into the human red cell were udied by means of a photoelectric densi-neter in most of the present investigations. he reliability and high accuracy of this nstrument was previously established by lawe ('56) who compared the rates of lucose penetration into the human ery-nrocyte by two methods, the photoelectric ensimetric technique and a chemical halytical method. However, the densi-neter is only useful for detecting substan-al cell volume changes produced by hy-ertonic solutions containing more than 1 M concentrations of a penetrating non-lectrolyte in isotonic saline. Therefore, n some of these investigations an analyti-al chemical method was used to study the ates of penetration of glucose into red ells suspended in buffered 0.006 M (ap-roximately 100 mg %) glucose-saline edia at various pH units.

Densimetry

The densimeter used in these experiments is an adaptation of the instrument developed by Ørskov ('35) and Parpart ('35) and very similar to the one used by Mawe ('56).

Washed blood (male, Rh positive, Type O) was used for all the densimeter measurements. It was prepared without using anti-coagulants in the following manner. Approximately 5 drops of whole blood, obtained by a lancet finger puncture, were suspended in 12 to 14 ml of buffered 1% NaCl and centrifuged. The supernatant was removed until its volume equaled that of the packed red cells. The cells were resuspended and washed two more times. Finally, the washed red blood cell suspension, consisting of an equal volume of cells and buffered 1% NaCl, was placed in a glass stoppered vessel which was rolled in an ice bath as the experiments proceeded.

The procedure for washing the whole blood took approximately 20 minutes which was enough time for the red cells to equilibrate to the pH of their environment. Jacobs and Parpart ('31) have demonstrated that the volume of a red cell is greatest at an acid pH and this volume decreases when the pH of the medium is increased. Therefore, a fresh sample of blood must be drawn and washed whenever an experiment is performed at a different pH.

For reproducible results the red cell suspension should not be more than two or three hours old. Bang and Ørskov ('37), Meldahl and Ørskov ('40) and Mawe ('56) have also observed that glucose penetrates into human red cells more slowly when the blood is not fresh.

In all of these experiments a standard phosphate buffer (Na_2HPO_4-NaH_2PO_4) which has a pK of 6.8 was used to buffer the solutions, (Parpart et al., '47). However, a glycyl-glycine buffer, pK 8.1, (Tyler and Horowitz, '37) and a bicarbonate-carbonic acid buffer, pK 6.2, (Henderson, '28, p. 44) were also used to buffer the glucose-saline solutions in order to detect any effect that may possibly be produced by phosphate ions.

The solutions were prepared in the following manner. Stock solutions of 1% and 2% NaCl were prepared. These solutions

were buffered to a desired pH with a standard phosphate buffer. When the other buffers were used, solid glycyl-glycine or bicarbonate was added to make a 0.02 M buffer solution. The pH desired was obtained in the 0.02 M bicarbonate-saline solutions by bubbling gaseous CO_2 into them; and 1 N NaOH was used to adjust the pH of the 0.02 M glycyl-glycine saline solutions. A glass electrode pH meter was used to measure the pH of these solutions.

Dry monosaccharides or sugar derivatives were then added to 50 ml of the buffered 1% NaCl. The amount of sugar or sugar derivative added depended upon the concentration at which the rate of penetration was being studied. These buffered sugar-saline solutions were not used experimentally until mutarotation had been completed (about two hours).

Precisely 20 mm^3 of a fresh, washed blood suspension were added to 10 ml of a sugar or sugar derivative buffered saline solution in the densimeter's suspension chamber. The red cells had been previously equilibrated to the same pH as the solutions to which they were added.

In order to measure the rates of penetration of the α- and β-isomers of D-glucose, 0.54 grams of dry α- or β-D-glucose was added directly to 10 ml of buffered 1% NaCl-PO_4 in the densimeter's suspension chamber. To this freshly prepared 0.3 M glucose-saline solution 20 mm^3 of a red cell suspension were added and the rate of penetration of the glucose isomer into the red cells was immediately measured. In some glucose-isomer experiments, 99.5% deuterium oxide was substituted for H_2O.

A Rudolph and Sons polarimeter, model 62, with a one decimeter temperature-controlled polarimeter tube and a General Electric sodium lab-arc were used to measure the specific rotations of the sugars.

The densimeter is calibrated with buffered 1% and 2% NaCl red cell suspensions before the rate of penetration of a substance is measured. The intensity of light transmitted by these suspensions, which is related to the volume of the red cells, is used as a criterion for the calibration of the densimeter. After the densimeter is calibrated with the 1% and 2% NaCl red cell suspensions, another 20 mm^3 aliquot of washed cells is added to a similarly buffered sugar or sugar derivative 1% NaCl solution. The initial decrease in the volume of the cells is in the direction of the light transmission previously recorded for the buffered 2% NaCl red cell suspension. However, the volume of the red cells gradually increases as the sugar or sugar derivative penetrates and eventually the amount of light that is transmitted by this suspension becomes the same as the amount of light that was transmitted by the 1% NaCl red cell suspension. The half-time for the achievement of this diffusion equilibrium was taken as a measure of penetration rate.

Chemical analytical

Normal human blood (approximately 5 ml) was drawn by venipuncture and washed three times with 1% NaCl-PO_4 at either pH 6, 7 or 8. This suspension was then placed in a glass stoppered flask and rolled in an ice bath. During the experiment, hematocrit determinations were made on the suspension (by the method of Parpart and Ballentine, '43) to detect any change that might occur in the ratio of cell volume to supernatant volume.

The rate of glucose penetration into human red cells was measured by determining, at various time intervals, the amount of glucose disappearing from the red cell medium. This was accomplished by the addition of a concentrated glucose-saline solution (containing completely mutarotated glucose) to seveal aliquots of the original 5 ml red cell suspension. Each aliquot of washed cells was exposed to the glucose for a designated length of time before it was centrifuged. Analysis of the supernatant revealed the loss of glucose that had occurred within a known time. The half-time to a diffusion equilibrium could easily be calculated from the relation between the amount of glucose that had disappeared from the supernatant and the length of time that the cells were exposed to glucose.

The procedure for this experiment is as follows. A 0.75 ml aliquot of the 5 ml red cell suspension is placed in an air turbine centrifuge tube and centrifuged (Parpart and Green, '51). After centrifugation 0.25 ml of the supernatant is removed and the remaining supernatant and light

cked red cells are mixed and equilib-ted to 37°C in a large oven. While is sample is being stirred at 37°C, 0.25 l of a glucose-saline solution (also 37°C and buffered to the same pH as e washed blood sample) is added to the be. The 0.25 ml glucose-saline solution of sufficient concentration to make the lls' environment 0.006 M (approximately 00 mg%) glucose in 1% NaCl-PO_4. This ucose-saline red cell suspension is stirred r a specific length of time while still at 7°C prior to separation of red cells and pernatant fluid. Before this centrifuga-on in the air turbine, 0.05 ml of n-butyl hthalate (free from butyric acid) is added the contents of the tube. The butyl hthalate is completely insoluble in water nd has a density less than that of the lls but greater than the glucose-saline lution. Consequently, it forms a layer etween the packed cells and their medium uring and after centrifugation. No glu-ose can penetrate into the cells after the yer of butyl phthalate has been formed. has also been established that butyl hthalate does not absorb or take up glu-ose under these conditions.

After this red cell suspension has been ntrifuged, 0.1 ml of the glucose-saline pernatant was placed into a test tube r analysis. A micro-analytical method, ith "Glucostat," a commercially prepared nzymatic glucose reagent manufactured Worthington Biochemical Corporation, reehold, New Jersey, was used for the uantitative analysis of glucose. This ethod is based on the specific enzymatic idation of glucose by glucose oxidase Saifer and Gerstenfeld, '58).

Investigation of mutarotase activity

Experiments were conducted to detect e possible existence of a mutarotase, an nzyme which facilitates the mutarotation either the α- or β-isomer of a sugar Keston, '54), which may be present in the asma membrane of the human red cell. Ghosts were prepared from human red ood cells by the method of successive molysis outlined by Hillier and Hoffman 53). A small quantity of these ghosts as added to a freshly prepared solution either α- or β-D-glucose. This suspension as placed in a temperature controlled (29°C) one decimeter polarimeter tube and its rate of mutarotation was recorded with a Schmidt and Haensch polarimeter. The rates of mutarotation of freshly prepared α- and β-D-glucose solutions, with and without ghosts, were compared.

Polarization microscopy

Mawe ('56) has suggested that sudden exposure of human red cells to an extremely hypertonic glucose-saline solution may cause a change in the physical state of the hemoglobin within the cells. These hemoglobin molecules may be in a paracrystalline state (Ponder, '45), which means that they could be more oriented and perhaps because of this the whole red blood cell may appear birefringent when observed with a polarization microscope.

If hypertonic glucose-saline solutions affect the physical state of the hemoglobin within red cells, then the osmotic behavior of the cells would be altered. Consequently, the measurement of cell permeability by osmotic methods under these conditions would be meaningless.

Approximately 2 ml of normal whole blood was defibrinated and centrifuged in the air turbine. The supernatant serum was divided into two equal portions. Enough dry glucose was added to one portion to make a 0.9 M glucose solution. A small amount of the packed red cells was added to the hypertonic serum containing glucose and to the normal control serum. These suspensions were examined in collaboration with Dr. R. D. Allen with an Inoué model polarization microscope soon after they were prepared. Fresh human serum was used to suspend the red cells because cells in a saline solution would crenate on a glass surface.

Source of materials

The distilled water used in these investigations was obtained from a still with a block-tin condenser tube leading to pyrex glass storage bottles. Deuterium oxide (heavy water) was purchased from the General Dynamics Coporation (Liquid Carbonic Division) in San Carlos, California.

Sodium phosphate (monobasic and dibasic), sodium chloride (for biological work), sodium bicarbonate and glucose

(anhydrous dextrose) were purchased from Merck and Company, Rahway, New Jersey.

The following reagents were purchased from the California Foundation for Biochemical Research, Los Angeles, California, D- and L-arabinose, D-fructose, D-galactose (anhydrous), D-ribose, L-sorbose and L-xylose.

D-xylose was obtained from the Pfanstiehl Chemical Company, Waukegan, Illinois.

D- and L-arabitol, β-D-glucose, D-mannitol and D-sorbitol were purchased from the Mann Research Laboratories, New York, New York.

The β-isomer of D-glucose was also obtained from Merck's dextrose (pure α-D-glucose) by crystallization from hot acetic acid and recrystallization from water and alcohol at lower temperatures according to the method of Hudson and Dale ('17). This highly purified β-D-glucose was used to verify the results obtained with the commercial product.

Glycyl-glycine was obtained from the Sigma Chemical Company, St. Louis, Missouri.

N-butyl phthalate was purchased from the Eastman Kodak Company, Rochester, New York and purified in the laboratory to remove traces of butyric acid.

RESULTS

Effect of pH and temperature upon the rates of sugar penetration from isosmotic concentrations prepared with isotonic salt solutions

The influence of pH on the rate of penetration of D-glucose into human red cells was first established and then thoroughly investigated before other sugars were tested. The half-time penetration to a diffusion equilibrium in completely mutarotated 0.3 M D-glucose dissolved in an isotonic NaCl-phosphate buffer at 37°C was found to be 23 minutes at pH 6, 8.5 minutes at pH 6.5, 1.5 minutes at pH 7, 2.5 minutes at pH 7.5 and 11.5 minutes at pH 8. The optimum pH range in which this sugar penetrates more rapidly is between pH 7 and pH 7.5.

The effect of pH upon the rate of glucose penetration is reversible. This was determined by many experiments in which re cells were washed three times in NaC PO_4 at a particular pH and then once mor at a different pH. Glucose penetration wa measured at the latter pH. In all the case examined, the rate of glucose penetratio corresponded with the pH at which th cells were finally equilibrated rather tha at any of the previous equilibrations higher or lower pH.

Variations in pH that were produced b a glycyl-glycine buffer and a bicarbonat carbonic acid buffer affected glucose pen tration in exactly the same manner tha was observed with the phosphate buffe Consequently, the recorded differences i the rates of glucose penetration at variou pH's can only be attributed to a fluctu tion in pH and not to an effect that may b produced by a specific type of buffer sy tem. However, the phosphate buffer ha a far better buffering capacity within th pH range 6 to 8 than has either the glycy glycine buffer or the bicarbonate-carbon acid buffer. Therefore, only the phosphat buffer was used throughout the remainde of these investigations.

Figure 1 shows the effect of pH upo the rate of penetration of 9 sugars (mea ured with the photoelectric densimeter at 29°C and 37°C. All the sugars pen trate more rapidly at pH 7. However, 29°C the sugars penetrate equally slow at pH 8 and pH 6, whereas at 37°C th sugars penetrate more slowly at pH 6 tha at pH 8. The sequence of sugars in the order of increasing rate of penetration pH 7 is: L-xylose, D-fructose, D-arabinos D-ribose, L-sorbose, D-xylose, D-galactos D-glucose and L-arabinose. It should b pointed out, however, that the only maj deviation from this pattern is exhibite by glucose at pH's acid and alkaline pH 7. From figure 1 it is also obvious th the human red cell shows a high degr of stereochemical specificity to monosa charides, but there is no correlation b tween the L- or D- forms of a sugar (whic are based on the configurational relatio between sugar molecules and L- or D-gly eraldehyde; Pigman, '57) and the suga rate of penetration.

The effect of temperature upon mon saccharide penetration, in relation to p is illustrated in table 1. The Q_{10} valu

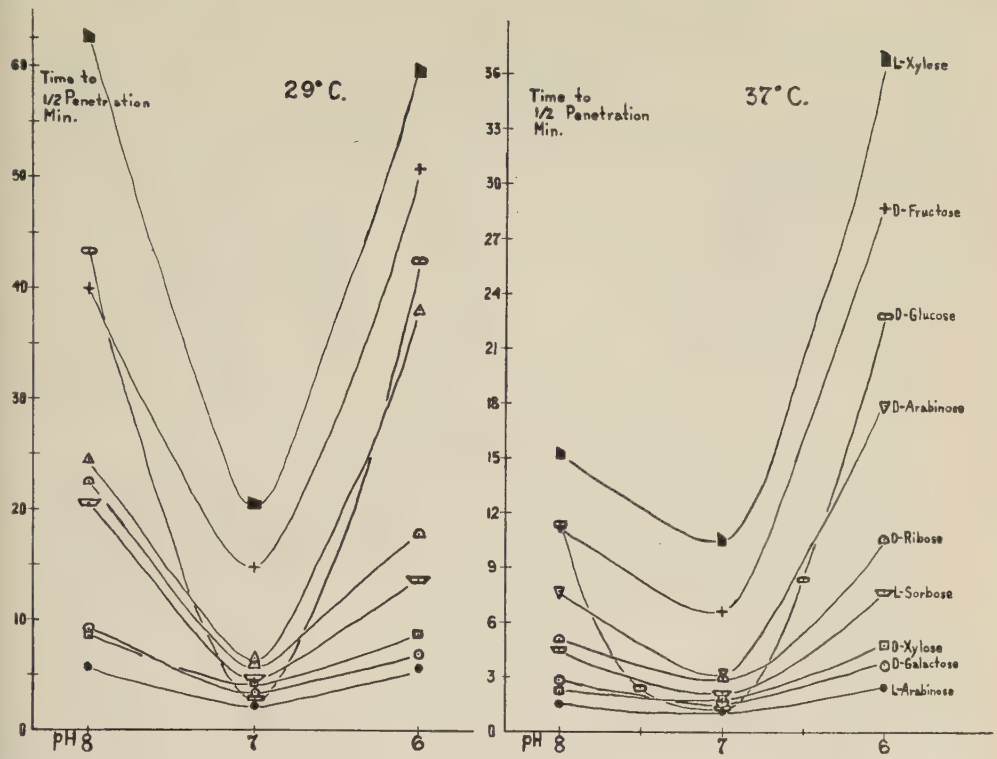

Fig. 1 Relative rates of penetration of various 0.3 M monosaccharides in 1% NaCl-PO_4. (Points represent an average of at least 5 determinations).

temperature coefficients) have been calulated from the rate of sugar penetration t 37°C and 29°C. It is interesting to note hat the Q_{10} values for all the sugars at pH's and 7 are between 2 and 2.8. At pH 8, here is a pronounced decrease in the rate f monosaccharide penetration when the emperature is dropped 8°C. Consequently, he Q_{10} values at pH 8 range from .1 to 6.5. No hexose or pentose tested had Q_{10} of less than 2 within the pH range xamined. Incidentally, the Q_{10} found for lucose penetration at pH 7 is approxiately the same as the value of 2.5 reorted by Bjering ('32) and LeFevre ('48) nd of 2.7, reported by Reinwein, Kalman nd Park ('57).

Unlike the sugar alcohols, no correlation xists between the number of carbon atoms n a sugar molecule and its rate of penetration. As shown in table 2, the 6 carbon sugar alcohols D-mannitol and D-sorbitol do not penetrate into the human red ell. However, the 5 carbon sugar alcohols D-arabitol and L-arabitol do penetrate, but at a much slower rate than their related sugars D-arabinose and L-arabinose. This slow penetration of the sugar alcohols (whose molecules are in a straight chain configuration) may serve to indicate that the sugar molecules have many of their -OH groups either in the ring or masked and thus act in a less polar manner.

Jacobs, Glassman and Parpart ('35) have demonstrated with other polyhydric alcohols that their rates of penetration into human red cells are determined by the number of carbon atoms in these compounds. According to their data ethylene glycol (two carbons) penetrates more rapidly than either glycerol (three carbons) or erythritol (4 carbons) and glycerol penetrates more rapidly than erythritol.

The effect of pH upon the rates of penetration of the 5 carbon sugar alcohols is also illustrated in table 2. These sugar alcohols penetrate rapidly and at the same rate at pH7 and their rates of penetration

TABLE 1

Results of Q_{10} studies on sugar penetration at pH 6, 7 and 8

Penetration data obtained from figure 1

Sugar	Q_{10} calculated from rates of penetration at 29°C and 37°C		
	pH 6	pH 7	pH 8
D-Glucose	2.0	2.2	5.3
L-Xylose	2.0	2.3	6.2
D-Fructose	2.1	2.7	5.0
D-Arabinose	2.5	2.7	4.3
D-Ribose	2.0	2.4	6.5
L-Sorbose	2.1	2.5	5.6
D-Xylose	2.0	2.8	5.0
D-Galactose	2.1	2.3	4.1
L-Arabinose	2.5	2.4	6.1

TABLE 2

Penetration of some sugar alcohols

Temperature 37°C; pH 6, 7 and 8; concentration 0.3 M

Alcohol	Number of carbon atoms	½ time penetration (minutes)		
		pH 6	pH 7	pH 8
D-Mannitol	6	∞	∞	∞
D-Sorbitol	6	∞	∞	∞
L-Arabitol	5	64.0	22.7	63.0
D-Arabitol	5	56.3	26.1	40.0

are similarly slowed at pH 6 and pH 8. However, at pH 8 L-arabitol penetrates approximately 1.5 times more slowly than D-arabitol. The situation is quite different in the case of the related sugars. At all pH's investigated, L-arabinose is the fastest sugar to penetrate into the human red cell, whereas D-arabinose is among the slowest (fig. 1).

Sugar penetration and specific optical rotation

Table 3 illustrates the close relationship that does exist between the observed relative rates of monosaccharide penetration and the specific optical rotation of the sugar molecules at pH 7. It is evident from these data that sugars with a positive specific rotation (dextrorotatory) penetrate more rapidly than sugars with a negative specific rotation (levorotatory) irrespective of the number of carbon atoms in the molecule. At pH 6 and 8, D-glucose is an exception, as illustrated in table 4 (pH 6) and table 5 (pH 8).

Since glucose penetration is more markedly affected by changes in pH than any of the other sugars that have been tested, it is possible that its mechanism for penetration is more complex. Therefore, mor extensive investigations with this sug were performed.

Concentration effect upon glucose penetration at pH 6, 7 and 8

Figure 2 shows the rates of penetratio at pH 6, 7 and 8 (37°C) of 0.006 M (10 mg%) to 0.8 M concentrations of glucos There is no apparent effect produced b pH upon the rate of penetration of 0.006 M glucose that could be determined with th previously described chemical analytic method. However, with the photoelectr densimeter, it has been demonstrated tha glucose penetration becomes progressivel slower at pH 6 and pH 8 when the co centration of glucose is increased from 0. M to 0.6 M. The rate of glucose penetr tion at pH 7 is not altered until the concen tration of glucose becomes greater tha 0.3 M (which is isosmotic for the huma red cell). At the concentration of 0.1 M the rates of glucose penetration are ver nearly the same at pH 7 and pH 8, but a pH 6, glucose penetrates a little slowe than at the other pH's.

Actually there is only a small differenc in the rate of glucose penetration at pH

TABLE 3

Relationship between specific rotation and sugar penetration at pH 7 and 37°C

Sugars in order of increasing penetration; specific rotations of sugars measured in 1% NaCl-PO_4

Sugar	Number of carbon atoms	½ time penetration at pH 7 and 37°C	Specific rotation at pH 7 and 37°C
		minutes	*degrees*
L-Xylose	5	10.6	−20.4
D-Fructose	6	6.7	−80.7
D-Arabinose	5	3.1	−99.1
D-Ribose	5	3.0	−17.7
L-Sorbose	6	2.2	−43.0
D-Xylose	5	1.8	+20.4
D-Galactose	6	1.7	+77.7
D-Glucose	6	1.5	+53.0
L-Arabinose	5	1.1	+98.7

TABLE 4

Relationship between specific rotation and sugar penetration at pH 6 and 37°C

Sugars in order of increasing penetration; specific rotations of sugars measured in 1% NaCl-PO_4

Sugar	Number of carbon atoms	½ time penetration at pH 6 and 37°C	Specific rotation at pH 6 and 37°C
		minutes	*degrees*
L-Xylose	5	34.0	−21.3
D-Fructose	6	29.0	−83.3
D-Glucose	6	23.0	+51.9
D-Arabinose	5	18.0	−99.1
D-Ribose	5	10.6	−18.7
L-Sorbose	6	7.7	−43.0
D-Xylose	5	4.9	+19.6
D-Galactose	6	3.8	+74.8
L-Arabinose	5	2.6	+98.0

TABLE 5

Relationship between specific rotation and sugar penetration at pH 8 and 37°C

Sugars in order of increasing penetration; specific rotations of sugars measured in 1% NaCl-PO_4

Sugar	Number of carbon atoms	½ time penetration at pH 8 and 37°C	Specific rotation at pH 8 and 37°C
		minutes	*degrees*
L-Xylose	5	15.2	−20.6
D-Glucose	6	11.5	+53.3
D-Fructose	6	11.3	−83.3
D-Arabinose	5	7.7	−100.0
D-Ribose	5	5.2	−17.7
L-Sorbose	6	4.3	−43.0
D-Galactose	6	2.9	+78.1
D-Xylose	5	2.3	+20.0
L-Arabinose	5	1.4	+98.7

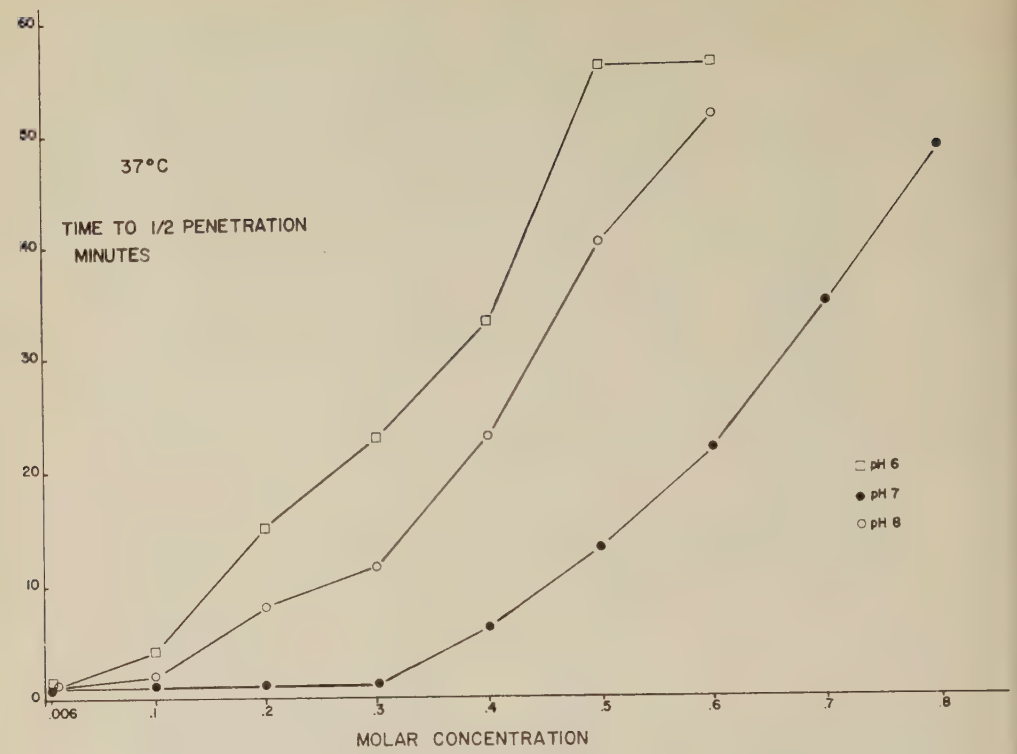

Fig. 2 The effect of concentration in relation to pH on the penetration of mutarotated D-glucose. (Points represent an average of at least three determinations).

over the concentration range of 0.006 M (half-time penetration of 0.5 minutes) to 0.3 M (half-time penetration of 1.5 minutes). The observed decrease in rate of glucose penetration at concentrations greater than 0.3 M may be attributed to the inability of the red cells to behave as true osmometers. A change in the physical state of the hemoglobin within these red cells could greatly affect the red cells' ability to undergo normal volume changes.

Polarization microscopy

Human red cells suspended in their own serum are not birefringent. Even when these cells were suspended in 0.9 M glucose-serum solution, no appreciable birefringence could be detected within these cells. In fact, washed human red cells suspended in a 0.9 M glucose-buffered saline solution (pH 6) that became crenated when placed on a glass surface because of the absence of serum proteins, also did not show any appreciable birefringence. Although Ponder ('44) regards crenation as one stage in the formation of intracellular crystalline hemoglobin and claims that crenated cells show more birefringence than normal cells, this could not be verified in these experiments. Even a small degree of orientation of the hemoglobin molecules that may have been produced within the crenated red cells or the cells exposed to hypertonic glucose-serum solutions would be detected with the Inoué model polarization microscope. However, it is possible that the osmotic properties of red cells suspended in hypertonic glucose-saline solutions may be altered without a subsequent change in the physical state of their intracellular hemoglobin.

pH and temperature effects on the relative rates of penetration of the α- and β-isomers of D-glucose

Mutarotated monosaccharide solutions are not homogeneous. These solutions con-

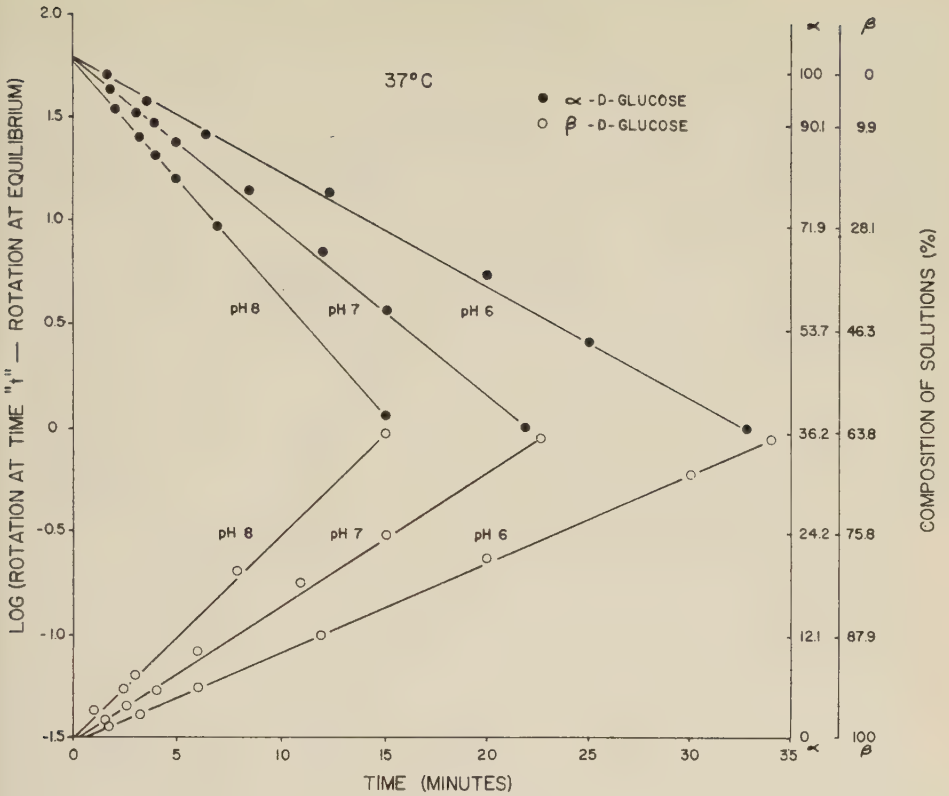

Fig. 3 Mutarotation of 0.3 M α- and β-D-glucose in 1% NaCl-PO_4 at pH 6, 7 and 8.

in α- and β-isomers which may be in a ranose or a furanose ring form. Although it would be advantageous to study e rates of penetration of each component an equilibrated sugar solution, this is npossible because some sugar anomers ave not been isolated and others have an xtremely rapid mutarotation velocity. owever, D-glucose is practical to use in n investigation of this type because its and β-isomers can readily be obtained a pure crystalline state which consists of ly the pyranose form. The mutarotation α- and β-D-glucose obey the first order action equation which makes it probable at the main constituents of their equilium solutions contain only the α- and pyranose modifications, (Pigman, '57, p.). Furthermore, the isomers do not murotate very rapidly in relation to their lf-time for penetration. This is shown figure 3 where the log (optical rotation a specific time "t"—optical rotation at equilibrium) vs. time is plotted for freshly prepared 0.3 M α-D-glucose and β-D-glucose dissolved in 1% NaCl-PO_4 at pH 6, 7 and 8 (37°C). The curves for the rates of mutarotation of these isomers are linear at each pH. The slopes of these curves agree with the information published by Hudson ('03) who had demonstrated that the rate of mutarotation of glucose is at a minimum between the pH limits 3 to 7. At pH values greater than 7 and less than 3, the velocity increases rapidly. The percentages of α- and β-glucose present in these various solutions at any particular time is also shown in figure 3. The actual composition of these solutions at time "t" can easily be determined because the composition of both the α- and β-isomer equilibrium solutions is 36.2% α-glucose and 63.8% β-glucose. These percentages are based on the equilibrium value of + 52.7 which is the specific rotation reached by both the pure α- and β-isomer.

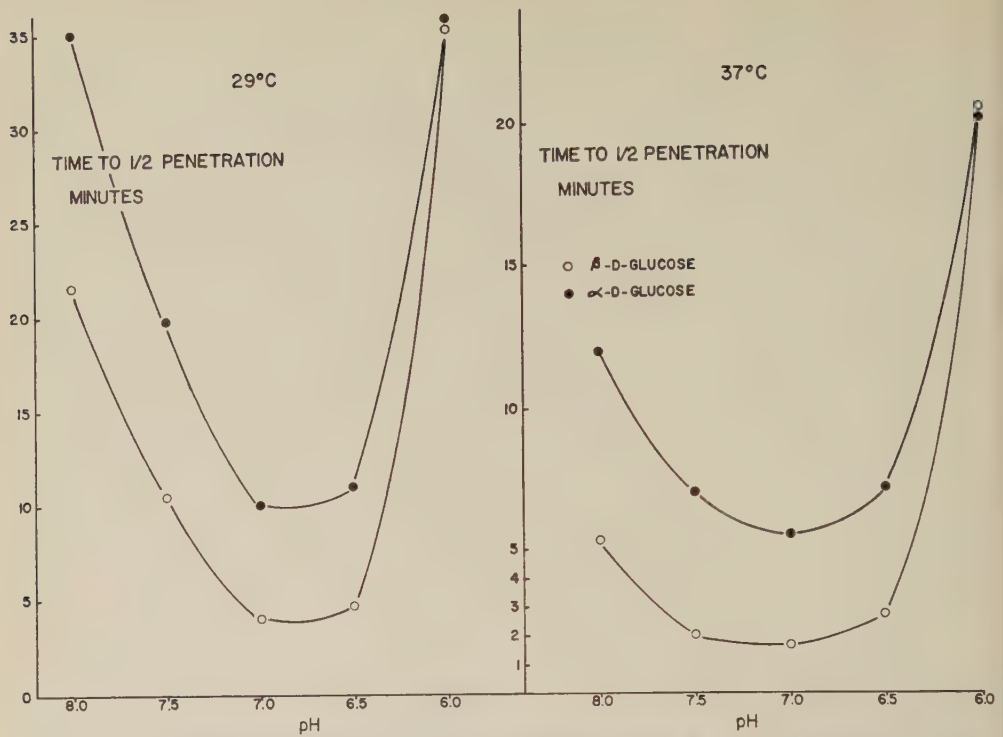

Fig. 4 A comparison between the rates of penetration of 0.3 M α- and β-D-glucose in relation to pH and temperature. (Points represent an average of at least three determinations).

Figure 4 shows the rates of penetration of 0.3 M α- and β-D-glucose at 29°C and 37°C in relation to changes in pH. The β-glucose penetrates approximately 3 times faster than α-glucose at pH 7 and 8. At pH 6 α- and β-glucose penetrate at the same rate. The optimum pH range for α- and β-glucose penetration at 37°C is between pH 7 and 7.5, but at 29°C the optimum range shifts to between pH 7 and 6.5. These measurements were obtained with both the commercially prepared β-glucose and with β-glucose prepared and purified in the laboratory from commercial α-glucose.

The actual composition of the initially pure α- and β-isomer solutions at their time of half penetration may be calculated from figure 3. One minute, which was the time allotted for each isomer to dissolve before its penetration was measured, should be added to the half-times to penetration before these percentage values are determined. At pH 6, 7 and 8 (37°C) the percentage of α-glucose present at the time to half penetration of the initial pure α-glucose solution is 60%, 81% an 54% respectively, whereas the percentag of β-glucose present at the time to ha penetration of the initially pure β-gluco solution is 78%, 96% and 84% respe tively.

In table 6 are shown the Q_{10} values ca culated from the rates of α- and β-gluco penetration in relation to changes in p at 29°C and 37°C. The inhibitory effe produced by this decrease in temperatu is greater for β-glucose than α-glucose pH 7, 7.5 and 8. However, at pH 6 an 6.5 the Q_{10} values for both isomers a the same.

The observations that β-glucose pen trates more rapidly than α-glucose at p 6.5, 7, 7.5 and 8 and that the temperatu coefficients for the β-isomer are somewh higher than those for the α-isomer at p 7, 7.5 and 8 indicates that perhaps son additional process or "factor" is requir for β-glucose penetration.

TABLE 6

Results of Q_{10} studies on α- and β-D-glucose penetration at pH 6, 6.5, 7, 7.5 and 8
Penetration data obtained from figure 4

Isomer	Q_{10} calculated from rates of penetration at 29°C and 37°C				
	pH 6	pH 6.5	pH 7	pH 7.5	pH 8
α-D-glucose	2.0	1.8	2.2	3.6	3.5
β-D-glucose	2.0	1.8	3.4	8.4	6.5

TABLE 7

The effect of deuterium oxide on the rates of penetration of 0.3 M α- and β-D-glucose
Temperature 37°C; pH 6, 7 and 8

Isomer	½ time penetration (minutes)					
	pH 6		pH 7		pH 8	
	H_2O	D_2O	H_2O	D_2O	H_2O	D_2O
α-D-glucose	20.0	30.0	5.5	6.1	12.0	12.2
β-D-glucose	20.4	27.8	1.5	5.9	5.3	11.5

Mutarotase activity

The α- and β-pyranose isomers of D-glucose differ only with respect to the spacial position of the hydroxyl group on the first carbon. Therefore, it may be possible to explain the differences in the rates of penetration of these isomers by postulating the presence of a mutarotase enzyme which in some manner abets β-glucose penetration. An investigation of the mutarotase activity in human red cell ghosts has indicated that these membrane preparations do not increase the rates of mutarotation of either freshly prepared α- or β-glucose solutions. This confirms the report by Reinwein, Kalman and Park ('57) who also did not find mutarotase activity in membrane preparations or in whole cells.

α- and β-glucose penetration in deuterium oxide

In table 7 data are presented on the rates of penetration of 0.3 M α- and β-glucose when dissolved in H_2O and D_2O at pH 6, 7 and 8 (37°C). The rate of penetration of α-glucose whether dissolved in D_2O or H_2O is affected in the same way at pH 7 and 8. At pH 6, however, it penetrates 50% slower when dissolved in D_2O.

On the other hand, β-glucose dissolved in H_2O penetrates more rapidly than α-glucose at pH 7 and 8, while at pH 6 there is no difference in the rates of their penetration. Table 7 also shows that β-glucose dissolved in D_2O penetrates less rapidly than when dissolved in H_2O at pH 7 and 8. It is therefore evident that in some manner the rate of penetration of β-glucose becomes the same as that of α-glucose when the former is dissolved in D_2O at pH 7 and 8.

DISCUSSION

The results of these investigations indicate that the mechanism of monosaccharide penetration into human red cells is not as simple as the postulated free diffusion mechanism, but on the other hand, it is not as complex as the active transport or the facilitated diffusion mechanisms. It will be argued that the data in this paper, as well as the results of experiments reported in the literature, substantiate the altered diffusion mechanism of sugar penetration into human red cells. This mechanism accounts for the highly selective properties of the plasma membrane by considering both the structure of the plasma membrane and the solubility properties, molecular size, spacial configurations and the degree of ionization of the sugars. Neither the free diffusion, the active transport nor the facilitated diffusion mechanisms have accounted for all of these factors.

Parpart and Ballentine ('52) have proposed, on the basis of chemical analytical and electron microscopy data, that the plasma membrane of the red cell is bas-

ically composed of an interlacing meshwork of protein (stromatin). Interspersed in this meshwork pattern are pores containing lipid molecules which are either bound to each other or to the protein molecules. They further postulated that water exists in the vicinity of the protein and the polar regions of the phospholipid molecules as aqueous channels which are continuous from the external environment to the interior of the cell. In addition to these aqueous channels, water pools may also exist in the membrane, Schmitt and Palmer ('40). Evidence has been presented by Jacobs et al. ('35) to support this hypothesis. The latter have shown that water penetrates the red cell with great rapidity and exhibits a temperature coefficient corresponding to a diffusion process.

According to the altered diffusion mechanism, the aqueous channels are the major avenues available to the lipid insoluble sugar molecules for penetration into the human red cell. The water pools in the plasma membrane are surrounded by lipid molecules and are probably not accessible to monosaccharides. Since the protein and phospholipid molecules in the pores of the plasma membrane are capable of forming hydrogen bonds with water (Bernal and Fowler, '33; Blanchard, '40; Klotz, '58 and Pauling, '60), it is conceivable that the water nearest to these molecules may be in a bound state and unavailable as a solvent to the diffusing sugar molecules. It is also conceivable that the aqueous channels in the pores of the plasma membrane are not straight routes from the external environment to the interior of the cell, but they may be convoluted or spiral in shape. The spacial relation of the protein molecules and the phospholipid molecules in the pores would impart this specific shape to the aqueous channels. Furthermore, the internal dimensions of these channels could be governed by the bound water.

By definition, the free diffusion mechanism does not explain the pH effect on the rates of penetration of various isosmotic sugars, the high temperature coefficient associated with their penetration and the stereospecificity observed with sugars in these investigations. However, the observations that sugars do not pene trate against a concentration gradient an that their rates of penetration, at an isos motic concentration, obey Fick's law c diffusion are valid and can not be dis regarded (Mawe, '56).

In the hypothesized active transport an facilitated diffusion mechanisms, it ha been postulated that either an enzyme (i the former case) or a "carrier" (in th latter case) form a complex with the suga molecule, thereby transporting it throug the membrane and into the cell. If a enzyme or a "carrier" did exist, it woul not be lipid in nature because all of th known enzymes are proteins. Therefor it is logical to assume, from our knowledg of the molecular anatomy and the chem ical composition of the plasma membrane that both the sugar molecule and th hypothesized sugar complex would use th aqueous channels as a means of penetra ing into the human red cell. Consequently if one subscribes to the idea of continuou water channels in the plasma membrane then it is unnecessary to postulate an en zyme or a "carrier" to render the suga molecule in a membrane-diffusible for since the sugar itself is already in thi state.

The high structural specificity and th large temperature coefficients associate with sugar penetration into the huma red cell have been cited as evidence sup porting sugar transport. LeFevre an Davies ('51) have claimed that in the pH range 7.1 to 7.4, the aldoses, D-glucose D-galactose, D-mannose, D-xylose and L arabinose share the same "carrier" trans port system with the ketoses, L-sorbos and D-fructose. According to their hypoth esis, the aldoses penetrate more rapidl than the ketoses because they form weaker and more dissociated complex wit the "carrier." They have also indicate that the Q_{10} values for the rates of pene tration of these sugars was 3.0.

It has been reported in the present in vestigations that the rates of penetratio of various 0.3 M aldoses and ketoses wer slowed when the pH of the medium wa changed from pH 7 to either pH 6 or 8 At first, these observations indicated tha sugars might combine with a surface con stituent of the cell. When half-time to

diffusion equilibrium vs. pH is plotted, a typical bell shaped curve is obtained, (fig. 1), which could be taken as indicative of a pH effect on the formation and decomposition of an enzyme-substrate complex of the Michaelis-Menton type, (Neilands and Stumpf, '58, p. 174). However, further investigations on glucose penetration at the concentrations of 0.2 M, 0.1 M and 0.006 M showed that the effect produced by pH variation, (fig. 2), becomes progressively less, until there is no pH effect upon the rate of 0.006 M glucose penetration. If a "carrier" or an enzyme sugar complex is being formed, then this reaction should be pH-dependent, regardless of the number of sugar molecules present in the medium.

This pH effect on the rates of monosaccharide penetration could conceivably be explained by the altered diffusion hypothesis. It is possible that pH changes may vary the amount of water in the aqueous channels of the plasma membrane. Consequently, the area available for diffusion in these channels would be influenced and this would determine the rate at which sugar molecules penetrate. At pH 7 (29°C and 37°C) there may be a maximum amount of water present. A deviation from neutrality, either toward an acid or an alkaline pH, may reduce the amount of water that is available for diffusion. When this occurs, the rate of sugar penetration would be slowed. Temperature as well as pH may play an important role in determining the amount of water in the aqueous channels. It has been shown in figure 1 that at 37°C, sugars penetrate more rapidly at pH 7; on the other hand at pH 8 and 6, there is a pronounced decrease in their rates of penetration. However, at 29°C, sugars penetrate equally slowly at pH 8 and 6. On the basis of what has been previously postulated, it is logical to assume that an increase in temperature from 29°C to 37°C at pH 8 may cause proportionally more water to be available for the diffusion of sugars than at either pH 6 or 7.

The high temperature coefficient that accompanies sugar penetration does not necessarily mean that a chemical reaction is taking place. However, it is indicative of a barrier to diffusion which must be surpassed in order for the sugar molecule to penetrate into the cell. This resistance, which is represented by a high activation energy, is overcome directly by thermal agitation and is exponentially related to temperature (Danielli and Davson, '34).

The hypothesized spiral shaped aqueous channels, the possible occurrence of an attraction between the penetrating sugar molecules and the bound water in the aqueous channels (by van der Waals forces or hydrogen bonding) and the amount of bound water in the channels all may contribute toward a barrier to diffusion.

LeFevre and Marshall ('58) have claimed that their hypothesized "carrier" forms a more stable complex with aldoses that are in the particular pyranose "chair" conformation (Isbell and Tipson, '59). However, it is virtually impossible to relate the conformational specificities of the sugar molecules with their relative rates of penetration unless the rates of penetration of each component in the mutarotated sugar solution is known. As has been demonstrated in this paper, the rates of penetration of α- and β-D-glucose dissolved in an H_2O-saline medium at pH 7 and 8 are not the same. Although both the anomers of glucose are in the pyranose modification, this is not true of many other equilibrated sugar solutions which are composed of isomers in the pyranose and furanose forms (Pigman, '57, p. 53). Also LeFevre and Marshall ('58) have completely disregarded the specific rotations of the sugar molecules with respect to their relative rates of penetration.

It has been demonstrated in the present investigations that monosaccharides with a negative specific rotation penetrate into the human red cell more slowly than monosaccharides with a positive specific rotation. This phenomenon is also evident with the LeFevre and Marshall ('58) data when these sugars are represented in their order of increasing penetration (at pH 7.4) with the sign of the optical rotation of their mutarotated solutions; L-glucose (−), L-galactose (−), L-rhamnose* (+), L-fucose (−), D-fructose (−), D-arabinose (−), L-sorbose (−), D-lyxose (−), D-ribose (−), L-arabinose (+), D-tagatose* (−), D-xylose (+), D-galactose (+), D-

mannose (+), D-glucose (+), and 2-deoxy-D-glucose (+). The two sugars marked with an asterisk apparently have rates of penetration which do not conform with the sign of their optical rotations. These exceptions could be caused by a small change in the pH of the medium, incomplete mutarotation of the sugar solutions when tested and by the use of red cells that have been stored for a number of days in the refrigerator. L-rhamnose and D-tagatose should be reinvestigated with these factors in mind.

It has been hypothesized in the altered diffusion mechanism that the aqueous channels, which are utilized by sugars to penetrate into the human red cell, are convoluted or spiral in shape. One may imagine that these spiral channels have a right-handedness. Therefore it is conceivable to postulate, by virtue of this phenomenon, that sugar molecules with a positive specific rotation may enter and pass through these channels more readily than sugar molecules with a negative specific rotation. On the other hand, it is possible for red cells of other species to contain aqueous channels whose structure permits the levorotatory sugar molecules to enter and pass through more readily than the dextrorotatory molecules. The rabbit red cell may be an example of this type. Morgan et al. ('55) have shown, at a neutral pH, that the rates of penetration of various hexoses and pentoses into rabbit red cells are: (in their order of increasing penetration) D-glucose (+), D-galactose (+), D-mannose (+), D-xylose (+), D-fructose (−), and D-ribose (−).

Wilbrandt et al. ('47), Rosenberg and Wilbrandt ('52) and LeFevre and Davies ('51) have claimed that the decrease in the rates of sugar penetration into human red cells produced by an increase in the sugar concentration, indicates that an enzyme or a "carrier" on the surface of the cell is being inhibited by an excess of substrate. However, Mawe ('56) has demonstrated (with both a photoelectric densimeter and a chemical analytical method) that the rate of glucose penetration into human red cells is constant between glucose concentrations of 0.1 M to 0.4 M at 37°C and pH 7.78. He has also shown that the rate of glucose penetration wa slower when measured in greater tha 0.4 M glucose-saline solutions at 37°C an pH 7.78. Mawe ('56) explained this phe nomenon by suggesting that cells place in hypertonic glucose-saline solutions en ter a state of "osmotic shock" and thereb are prevented from behaving as tru osmometers.

The rates of penetration at pH 6, 7 an 8 (37°C) of 0.006 M (108 mg%) to 0.8 M glucose have been measured in the presen investigations (fig. 2). The only concen tration in which the rate of glucose pene tration was not affected by pH was 0.00 M, which is approximately the same as th normal blood sugar concentration (10 mg%). It has been previously postulate that at 37°C, any pH deviation from neu trality may decrease the amount of wate in the aqueous channels. The amount o this water available for diffusion may b the limiting factor associated with th rate of penetration of glucose at concen trations greater than 100 mg%. Conse quently, the ratio of water to the numbe of sugar molecules in the aqueous chan nels beyond the critical concentration o 0.006 M glucose, may determine the rat of glucose penetration.

Not only is the mount of water available for diffusion important, but one must also consider the possibility of an increased interaction by van der Waals forces, elec trostatic forces and hydrogen bonding be tween the penetrating sugar molecule and the inner wall of the aqueous channels in hypertonic sugar-saline solutions. All o these factors would contribute to a de crease in the rate of sugar penetration in hypertonic sugar solutions.

The optical polarization studies, per formed in collaboration with Dr. R. D Allen, on human red cells in these investi gations, did not reveal any appreciable birefringence in red cells exposed to hyper tonic glucose-serum or glucose-saline solu tions. However, it is still possible that the osmotic properties of these cells were al tered without any observable interna orientation or crystallization of their hemo globin. Therefore, in addition to the fac tors previously mentioned, an apparen decrease in the rate of sugar penetration in a hypertonic sugar-saline solution may

be produced by the inability of red cells to behave as true osmometers.

Wilbrandt ('50) has claimed that enzymes are involved in the penetration of sugars into human red cells because sugar permeation is reduced in the presence of enzyme inhibitors such as Hg^{++}, Hg^{+++}, p-chloromercuribenzoate, gold, chloropicrin, bromoacetophenone and allyl isothiocyanate which supposedly bind with the transport enzyme. LeFevre ('47), ('53), ('54) and ('59) believes that phlorhizin and phloretin inhibit sugar penetration by combining reversibly with the "carrier," in competition with the sugars and that inhibition by the mercuric ion does not fit into this pattern. The fact that these substances bind with the plasma membrane does not necessarily indicate that an enzyme or a "carrier" is being inhibited. These data can also be interpreted by postulating that the amount of water available for diffusion in the aqueous channels is reduced when these substances bind with the plasma membrane. Parpart et al. ('47) have shown that the rate of water penetration into human red cells is slowed when these cells have been previously exposed to p-chloromercuribenzoate and Hg. Any final statement concerning the mechanism of monosaccharide penetration into human red cells must account for the rates of penetration of each component in the mutarotated sugar solutions. It has been demonstrated in this paper that in an H_2O-saline medium, 0.3 M β-D-glucose penetrates more rapidly than 0.3 M α-D-glucose at pH 7 and 8 (37°C). The calculated Q_{10} values for β-D-glucose penetration at these pH's are higher than the Q_{10} values for α-D-glucose penetration. These data suggest that perhaps another factor, in addition to the hypothesized altered diffusion mechanism, is involved in β-D-glucose penetration. An investigation of mutarotase activity on the red cell membrane had indicated that this enzyme was not present. However, a study of the rates of penetration of α- and β-D-glucose in D_2O revealed that these isomers penetrate at the same rates in this medium. Therefore, it may be concluded that hydrogen bonding is an important factor involved in the penetration of β-D-glucose in an H_2O-saline solution at pH 7 and 8. The mechanism of β-D-glucose penetration may be a special case and requires further study.

Consideration of the results of this investigation makes it clear that monosaccharide penetration into human red cells by an altered diffusion mechanism fit the available data more adequately than either of the previously hypothesized mechanisms.

SUMMARY

1. The relative rates of penetration of 0.1 M and higher concentrations of monosaccharides (dissolved in buffered saline solutions) into human red cells were studied by means of a photoelectric densimeter. A chemical method was used to study the rate of penetration of 0.006 M glucose.

2. The effect of pH and temperature upon the rates of sugar penetration from isosmotic concentrations prepared with isotonic salt solutions was investigated. When studied at 29°C and 37°C, all the sugars penetrated more rapidly at pH 7. However, at 29°C the sugars penetrated equally slowly at pH 8 and pH 6, whereas at 37°C the sugars penetrated more slowly at pH 6 than at pH 8.

3. In all cases the final distribution of these mutarotated sugars between the red cells and their environment was one of diffusion equilibrium.

4. The pH effect on sugar penetration was reversible.

5. The Q_{10} values calculated from the rates of sugar penetration at 37°C and 29°C for all the tested sugars at pH's 6 and 7 were between 2 and 2.8. The Q_{10} values at pH 8 ranged from 4.1 to 6.5.

6. A close relationship between sugar penetration and the sign of specific rotation of the sugars has been observed. All the sugars with a negative specific rotation penetrated more slowly than sugars with a positive specific rotation irrespective of the number of carbon atoms in the sugar molecule.

7. There was no apparent effect produced by pH upon the rate of penetration of 0.006 M glucose that could be determined with a chemical analytical method. However, glucose penetration became progressively slower at pH 6 and pH 8 when

the concentration of glucose was increased from 0.1 M to 0.6 M.

8. The rate of glucose penetration at pH 7 was not appreciably altered until the concentration of glucose became greater than 0.3 M which is isosmotic to the human red cell.

9. No birefringence could be detected in red cells exposed to extremely hypertonic glucose-serum and glucose-saline solutions.

10. The rates of penetration of 0.3 M α- and β-D-glucose at 29°C and 37°C were studied in relation to changes in pH. The β-D-glucose penetrated approximately 3 times faster than α-D-glucose at pH 7 and pH 8. At pH 6, α- and β-D-glucose penetrated at the same rate.

11. No mutarotase activity was detected in membrane preparations of human red cells.

12. When 99.5% deuterium oxide was substituted for water, the α- and β-isomers of D-glucose penetrated at approximately the same rate at pH 7 and 8.

13. The results of these investigations and other data reported in the literature indicate that sugars may penetrate into human red cells by an altered diffusion mechanism.

14. The altered diffusion mechanism accounts for monosaccharide penetration into human red cells by considering the structure of the plasma membrane as well as the solubility properties, molecular size, spacial configurations and the degree of ionization of the sugars.

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